DEFICIENCIES OF CERTAIN VITAMINS AS STUDIED WITH TURKEY POULTS ON A PURIFIED DIET

I. PTEROYLGLUTAMIC ACID, RIBOFLAVIN, NIACIN AND INOSITOL

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SIX FIGURES

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Difficulties have been encountered with turkeys, as with chicks, when attempts have been made to devise purified diets which will permit normal growth. Now that synthetic pteroylglutamic acid is available (Angier et al., '46) it has been found possible by means of its inclusion to prepare diets in which the water-soluble vitamins are supplied in synthetic form and which give satisfactory growth in turkeys.

EXPERIMENTAL

Day-old turkey poults were placed in electrically heated battery brooders and were fed the experimental diets immediately. Ten or eleven Bronze or White Holland turkeys were used in each group.

In preliminary experiments glucose was used as the source of carbohydrate. Difficulties occurred, including stickiness and caking of glucose on the feathers, the occurrence of "pendulous crops" as early as 3 weeks of age, and high mortality. Since the first of these difficulties was thought to be due to the physical properties of glucose, a change was made to corn

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starch, and no further difficulties were encountered. The possibility remained that the starch may have contained some unidentified ingredient which was responsible in part for the improvement.

Fig. 1 Turkey on diet T-15 at 11 weeks of age, weight 2348 gm, and of comparatively normal appearance except for a slight enlargement of the crop.

The following basal diet was used: starch 55.5 gm, purified casein [1] 20 gm, gelatin 8 gm, calcium gluconate 5 gm, glucose [2] 0.88 gm, cystine 0.4 gm, choline chloride 0.2 gm, inositol 0.1 gm, bone ash 2 gm, NaCl 0.6 gm, KH_2PO_4 0.45 gm, K_2HPO_4 0.6 gm, $MgSO_4$ 0.25 gm, $MnSO_4$, 4 H_2O 0.05 gm, ferric citrate 0.05 gm, $CuSO_4$, 5 H_2O 2 mg, $Al_2(SO_4)_3$, 18 H_2O 1.6 mg, KI 0.6 mg, cobalt chloride 0.4 mg, nickel chloride 0.2 mg, calcium

[1] Labco.
[2] Cerelose.

pantothenate 5 mg, niacinamide 5 mg, riboflavin 1 mg, pyridoxine HCl 1 mg, thiamine HCl 1 mg, p-aminobenzoic acid 1 mg, 1-acetoxy-2 methyl-4 naphthyl sodium phosphate 0.5 mg, sodium pteroylglutamate 2 mg, (dl) biotin .04 mg, to which were added vitamin A 3000 U.S.P. units, vitamin D 400 A.O.A.C. units, mixed tocopherols 68 mg, dissolved in corn oil [3] to a total of 6 gm.

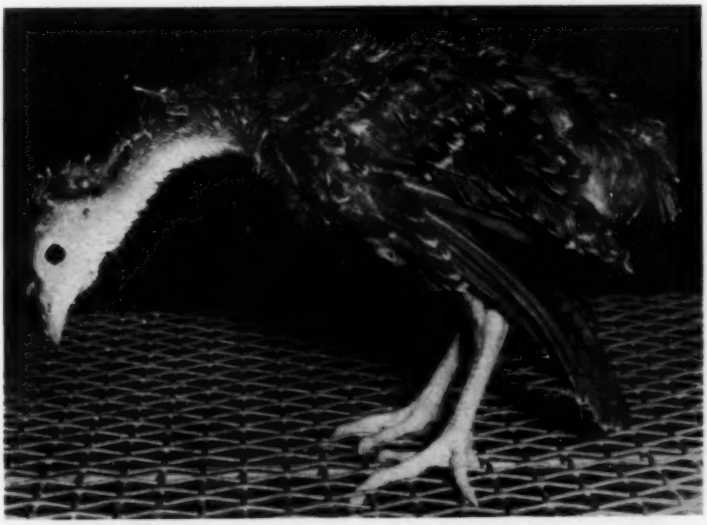


Fig. 2 Cervical paralysis in turkey in group 1, deficient in pteroylglutamic acid. Age 33 days.

The water-soluble vitamins were added as follows: inositol, niacinamide, calcium pantothenate, thiamine, riboflavin, pyridoxine, p-aminobenzoic acid and 1-acetoxy-2 methyl-4 naphthyl sodium phosphate were mixed dry with glucose so that 1 gm of the mixture was sufficient for 100 gm of diet. (dl) biotin was dissolved, 0.8 mg per ml, in 50% alcohol with the aid of a drop of ammonium hydroxide solution and the biotin solution was dried on the glucose before the other vitamins were mixed with it. Sodium pteroylglutamate was prepared by

[3] Mazola.

dissolving pteroylglutamic acid in just sufficient sodium hydroxide solution and making up to a final concentration of 2 mg of pteroylglutamic acid per ml in 50% ethanol. The solution was added to the diet. Turkeys on this diet ("diet T-15") appeared normal (fig. 1), and grew at a fair rate, although not at the maximum rate which might be anticipated. Blood was drawn from a wing vein for hemoglobin determinations. The blood was hemolyzed with dilute ammonium hydroxide and readings were made in the Coleman spectrophotometer at 540 mμ. The calculations were made from a standard curve which was constructed on the basis of a comparison of the readings obtained for a series of hemoglobin solutions with both the Evelyn colorimeter and the Coleman spectrophotometer. Cell volume determinations were made by centrifuging blood in standard Wintrobe hematocrit tubes. Smears were stained supravitally with brilliant cresyl blue and counterstained with Wright's solution.

Pteroylglutamic acid

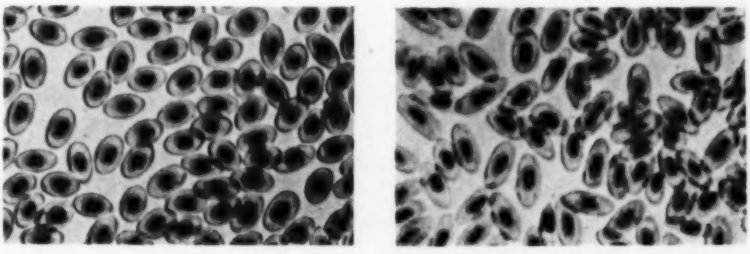
A deficiency of this vitamin resulted in slow growth and a moderate degree of anemia. The results are shown in table 1.

TABLE 1

Effect of various levels of pteroylglutamic acid on turkey poults fed a basal purified diet consisting of diet T-15 with pteroylglutamic acid omitted.

GROUP	SUPPLEMENT PER KILO OF DIET	WEIGHT IN GM AT			PER CENT HEMOGLOBIN AT		AT 4 WEEKS	
		21 days	28 days	42 days	3 weeks	4 weeks	RBC count	Mean corpuscular volume
1	None	165	191	all dead	8.65	10.42	2.43	1.12
2	0.2 mg pteroylglutamic acid	201	284	409	9.19	10.66	2.50	1.04
3	0.5 mg pteroylglutamic acid	194	287	565	9.46	10.04	2.71	0.99
4	0.8 mg pteroylglutamic acid	225	324	646	9.66	10.05	2.65	1.04
5	1.0 mg pteroylglutamic acid	200	315	607	9.35	10.23	2.68	1.06

Three cases of cervical paralysis (Richardson, Hogan and Kempster, '45) were observed among 4 surviving birds on the basal diet at 33 to 35 days of age (fig. 2). The results indicate that growth was somewhat better at a level of 0.8 mg of pteroylglutamic acid per kilo of diet than at a level of 0.5 mg. Increasing the level to 1.0 mg did not increase the growth rate. Examination of blood smears revealed a difference in appearance between the erythrocytes from turkeys



3A 3B

Fig. 3 A Normal-appearing erythrocytes (× 1500) from turkey in group 5, receiving a diet supplemented with 1.0 mg pteroylglutamic acid per kilo. B. Erythrocytes (× 1500) from turkey in group 1, deficient in pteroylglutamic acid. Note the large size and elongation of the cells as compared with the normal erythrocytes. Some of the cells show nuclei which are enlarged and of diminished density. The blood smears were made at 28 days of age.

in group 1 with pteroylglutamic acid deficiency and the erythrocytes from turkeys in group 5. The erythrocytes of the deficient birds were larger in diameter and their nuclei were larger and appeared less dense than in the case of the birds receiving supplementary pteroylglutamic acid (fig. 3).

Riboflavin

Omission of riboflavin from the basal diet resulted in slow growth and dermatitis as summarized in table 2. The dermatitis (fig. 4) was observed in the region of the eyes and mouth and corresponded closely to the original descriptions of riboflavin-deficiency dermatitis in turkeys (Lepkovsky and Jukes,

'36; Jukes, '38). The requirement for riboflavin under the conditions of the experiment appeared to be satisfied by 3.0 mg per kilo of diet. A level of 2.5 mg per kilo was insufficient for growth and did not give complete protection against dermatitis.

An additional group was included in the series. The group (group 9 A) received the same diet as group 9, but with casein [4] replacing gelatin in the diet. The object was to obtain

TABLE 2

Effect of various levels of riboflavin on turkey poults when added to a basal purified diet consisting of diet T-15 with riboflavin omitted.

EXP.	GROUP	SUPPLEMENT PER KILO OF DIET	WEIGHT IN GM AT			PER CENT OF BIRDS SHOWING DERMATITIS AT		
			21 days	28 days	42 days	17 days	25 days	42 days
1	6	None	134	170	210	56		80
1	7	2.5 mg riboflavin	199	254	457	25		0
1	8	3.0 mg riboflavin	228	340	610	0		0
1	9	4.0 mg riboflavin	226	338	640	0		0
1	9A	Same diet as group 9 but with casein replacing gelatin	187	260	404	0		0
2	19	None	105 [1]	117 [2]	150†	75	66 [2]	
2	20	2.5 mg riboflavin	139	152	198	11	44	57
2	21	3.0 mg riboflavin	120	150	210	11	33	25
2	22	4.0 mg riboflavin	168	244	429	0	22	11
2	23	10.0 mg riboflavin [3]	189	266	472	0	0	0

[1] 5 survivors † 1 survivor.

[2] 3 survivors.

[3] 1.2 mg pteroylglutamic acid per kilo of diet.

some indication as to whether turkeys, like chickens (Almquist and Mecchi, '40), require glycine in the diet for rapid growth. The results indicated that such was the case.

A second experiment with riboflavin was carried out with a group of turkeys which were hatched in August. The results of this experiment are also summarized in table 2 and indicate a higher riboflavin requirement than was observed in the first experiment with this vitamin. The turkeys in the

[4] Labco.

second experiment did not grow as rapidly on higher levels of riboflavin as did the turkeys in the first experiment and dermatitis appeared in groups 21 and 22 which received, respectively, 3.0 mg and 4.0 mg of riboflavin per kilo of diet. The slow growth which was obtained in experiment 2, and which was observed even in the case of group 23, may have been associated with the commonly observed fact that turkeys hatched late in the breeding season tend to grow more slowly and to exhibit less vitality than turkeys which are hatched at the height of the breeding season.

TABLE 3

Effects of various levels of niacinamide on turkey poults when added to a basal purified diet consisting of diet T-15 with niacinamide omitted.

GROUP	SUPPLEMENT PER KILO OF DIET	WEIGHT IN GM AT			PER CENT INCIDENCE OF PEROSIS AT		
		21 days	28 days	42 days	18 days	27 days	42 days
10	None	92 [1]	all dead	. .	0 [1]	all dead	. .
11	20 mg niacinamide	158	215	341	33	89	100
12	50 mg niacinamide	182	273	479	0	0	0
13	100 mg niacinamide	174	263	487	0	0	0

[1] One survivor.

Niacin

Without the addition of niacin to the diet, only 1 of 10 birds survived to the age of 18 days as indicated in table 3, group 10. Perosis (fig. 5) occurred in birds in group 11 which received the diet containing 20 mg of added niacinamide per kilo; this confirms the report by Briggs ('46). No abnormalities were observed in the birds in groups 12 and 13 which received 50 or 100 mg of added niacinamide per kilo of diet. Growth was more rapid in groups 12 and 13 than in group 11.

Inositol

The results with inositol are summarized in table 4. Growth was improved by adding 1.0 gm of inositol per kilo but not by 0.1 gm. The hemoglobin level was lowered somewhat by omitting inositol from the diet.

Fig. 4 Dermatitis and rough feathering in turkey in group 6, deficient in riboflavin. Age 21 days.

Fig. 5 Perosis in turkey in group 11, receiving a diet containing a suboptimal level of niacinamide. Age 42 days.

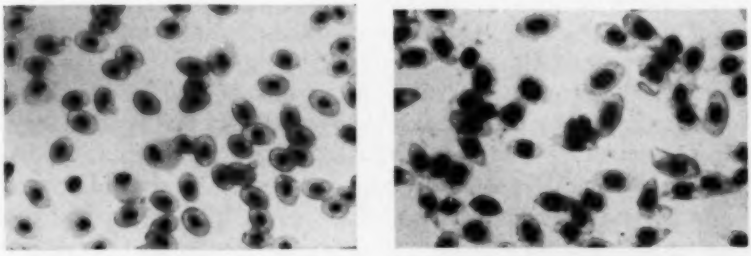
TABLE 4

Effect of various levels of inositol on turkey poults when added to a basal purified diet consisting of diet T-15 with inositol omitted.

EXP.	GROUP	SUPPLEMENT PER KILO OF DIET	WEIGHT IN GM AT			PER CENT HEMOGLOBIN AT		
			21 days	28 days	40 days	21 days	33 days	42 days
1	14	None	139	194	290	8.28	9.16	
1	15	0.1 gm inositol	141	192	271			
1	16	1.0 gm inositol	166	234	416	9.57	9.98	
2	17	None	137	171	221	9.18	10.32	10.30
2	18	1.0 gm inositol	159	209	322	11.30	10.37	12.24

An examination was made of stained blood smears from anemic turkeys in group 17 (table 4) at 6 weeks of age. The erythrocytes had an appearance which indicated a normocytic anemia, and which was characterized by approximately normal cell diameter, the cells round rather than oval in appearance, the nuclei enlarged and exhibiting irregular color density, and the cytoplasm staining a deeper blue than in normal cells. The erythrocytes in corresponding smears obtained from group 18 were normal in appearance (fig. 6).

The presence of inositol, as phytic acid, in avian erythrocytes was reported by Rapoport ('40). It was later noted



6A 6B

Fig. 6 A. Normal-appearing erythrocytes (× 1500) from turkey in group 18 on diet supplemented with 1 gm of inositol per kilo. B. Erythrocytes (× 1500), showing normocytic anemia, obtained from turkey in group 17, deficient in inositol. Note the paleness of the cytoplasm, the anisocytosis and the enlargement of the nuclei. Some immature red cells, characterized by darker cytoplasm and especially large nuclei, are present. The blood smears were made at 42 days of age.

(Rapoport and Guest, '41) that turkey blood contained 74.2 mg of phytic acid phosphorus per 100 ml of packed cells. Phytic acid was not detected by these investigators in the blood of 22 mammalian species.

DISCUSSION

The observation regarding the paralytic condition associated with pteroylglutamic acid deficiency in turkeys (Richardson et al., '45) has been confirmed in the present investigation. The present report indicates that the pteroylglutamic acid requirement of young turkeys may be at least twice that of chicks. Other investigations (Russell, W. C., '46, private communication) indicate that the pteroylglutamic acid requirement of young turkeys under certain conditions may be even higher than the levels studied in the present investigation.

In a previous investigation (Jukes, '38) it was noted the amount of riboflavin required in the diet of turkey poults was about the same as that required by chicks. The first experiment in the present report tends to support this finding. The second experiment, which was made with "late-hatched" turkeys, indicated a higher riboflavin requirement. The riboflavin requirement of young turkeys was investigated with a diet of natural foodstuffs and was estimated as 2.7 mg per kilo of diet (Patrick et al., '44). In a recent investigation, the riboflavin requirement of young turkeys appeared to be in the neighborhood of 3.5 mg per kilo of diet (Bird et al., '46). Dermatitis was noted which was attributed to biotin deficiency in the basal diet. Perosis was noted on suboptimal levels of riboflavin. These observations may be contrasted with another report (McGinnis and Carver, '46) in which riboflavin was found to be very effective in preventing dermatitis in young turkeys.

The niacin requirement appeared to be satisfied by 50 mg of niacinamide per kilo of ration but 20 mg was insufficient.

Inositol deficiency in turkeys has not previously been described. The deficiency resulted in growth and a normocytic anemia. A level of 1.0 gm of inositol per kilo of diet produced

more rapid growth than was produced by 0.1 gm. Levels higher than 1.0 gm were not fed.

The growth of turkeys was reduced by substituting casein for gelatin in diet T-15. This may possibly indicate that turkeys, like chicks (Almquist and Mecchi, '40), require glycine for growth.

SUMMARY

1. Young turkeys were found to grow and survive on a purified diet containing synthetic vitamin B complex factors.

2. Slow growth, cervical paralysis, a moderate degree of anemia, and high mortality were observed when pteroylglutamic acid was omitted from the diet. The requirement for pteroylglutamic acid under the conditions of the experiment appeared to be in the neighborhood of 0.8 mg per kilo of diet. A characteristic appearance of the erythrocytes in pteroylglutamic acid deficiency was observed and is illustrated.

3. Riboflavin deficiency resulted in slow growth and dermatitis. A level of 2.5 mg of riboflavin per kilo of diet was not sufficient for growth or protection against dermatitis, but 3.0 mg appeared to be sufficient for the first 6 weeks under the conditions encountered in 1 experiment. A higher requirement, apparently somewhat in excess of 4.0 mg of riboflavin per kilo of diet, was indicated in a second experiment which was made with birds hatched at the end of the breeding season.

4. Omission of niacin from the basal diet resulted in slow growth and high mortality. A level of 20 mg of added niacinamide per kilo of diet enabled the birds to survive but growth was slow and the turkeys developed perosis. A level of 50 mg of added niacinamide appeared to be sufficent for fairly rapid growth and for the prevention of perosis.

5. Inositol deficiency was found to produce slow growth and a normocytic anemia.

6. Substitution of casein for gelatin in the basal diet resulted in slow growth. This may indicate that turkeys, like chicks, require glycine in the diet.

ACKNOWLEDGMENTS

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PHOSPHORUS IN POULTRY NUTRITION

III. THE RELATIONSHIP BETWEEN THE SOURCE OF VITAMIN D AND THE UTILIZATION OF CEREAL PHOSPHORUS BY THE POULT [1]

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ONE FIGURE

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The observations of Bird ('44) and Boucher ('44) that vitamin D_3 is essentially two times as effective as vitamin D from cod liver oil in promoting calcification in poults offered a possible explanation for some of the variations which appear in the literature regarding the vitamin D requirements of the turkey poult. That this difference in efficacy might be associated with the mineral content of the diet is suggested by the data of Fritz, Hopper and Moore ('45), and more specifically with the phytin phosphorus by Matterson, Scott and Singsen ('46). According to Singsen and Mitchell ('45) when chicks were forced to rely upon phytin as their principle source of phosphorus, irradiated animal sterols were more effective in promoting calcification than was cod liver oil. The experiments reported here were conducted in an effort to demonstrate that with the poult, the relationship between the source of vitamin D and the utilization of phytin phosphorus is essentially the same regardless of whether calcium magnesium phytate or the natural cereal ingredients are used to supply the phytin.

[1] Supported in part by a grant from the Yantic Grain and Products Company, Norwich, Connecticut.

A preliminary report of this work was made at the Annual Meeting of the Poultry Science Assn. at Saint Louis, July 23, 1946. The abstract of the paper appears in Poul. Sci., *25:* 411, 1946.

Mussehl and Ackerson ('35) have shown that normal growth and bone ash may be obtained with turkeys when the levels of phosphorus intake vary from 0.63 to 1.47%. Hammond, McClure and Kellogg ('44) have shown that 0.62% phosphorus and 1.02% calcium would support excellent calcification in poults when 80 A.O.A.C. chick units of vitamin D per 100 gm of feed were fed. Unpublished data obtained at the Storrs Agricultural Experiment Station confirm these findings, in that 0.60% available phosphorus supports normal growth and calcification. Evans and Brant ('45) reported that 0.80% phosphorus, a calcium : phosphorus ratio of 2 : 1 and 100 A.O.A.C. chick units of vitamin D per 100 gm of diet supported excellent calcification. The vitamin D was supplied by what the authors call a "natural vitamin D oil." The diets used in the experiments reported here were planned to contain about 0.60% total phosphorus, a level which is close to the minimum requirement of the turkey poult.

The vitamin D requirements of the turkey poult have been investigated by a number of workers, and values may be found in the literature that range from as low as 80 to over 200 A.O.A.C. chick units per 100 gm of diet. Relatively low levels of vitamin D were chosen for use in our experiments in order to bring out as clearly as possible any differences in efficacy which might exist.

The objects of experiment 1 were, first, to investigate the effect of different levels of cereal phosphorus on bone calcification in the poult when the total calcium and phosphorus intakes and the level of vitamin D are held constant, and second, to measure the effect, if any, of the source of vitamin D on the utilization of cereal phosphorus.

The second experiment was designed to measure the effects on bone calcification (bone ash) of increasing unitages of vitamin D from cod liver oil and irradiated animal sterols (irradiated 7-dehydrocholesterol) with two diets which had been shown in experiment 1 to be rachitogenic because too large a proportion of the total phosphorus was contributed by cereal ingredients. It was our hope to be able to make specific

vitamin D recommendations for turkey poults depending upon (1) the source of vitamin D being used and (2) the total amount of and the sources of phosphorus in the diet.

MATERIALS AND METHODS

The basal diet used in these experiments minus the cereal ingredients and the calcium and phosphorus supplements, appears in table 1.

TABLE 1

Composition of the basal diet.

BASAL MIXTURE		VITAMIN SUPPLEMENT	
	%		*Per 100 lb. diet*
Casein	12.00	Calcium pantothenate	635 mg
Gelatin	3.00	Pyridoxine	227 mg
Primary grown yeast	4.00	Riboflavin	227 mg
Bagasse	4.00	Inositol	227 mg
Liver meal	3.00	Para-aminobenzoic acid	227 mg
Wheat germ oil	1.00	Thiamine	95 mg
NaCl	0.50	Nicotinic acid	95 mg
KCl	0.50	Vitamin K	18 mg
$MnSO_4$	(70 gm)	Choline chloride	45.4 gm

The diet was made up to 100% with additions of ground limestone, bone meal and special white corn meal (a de-germed, de-braned product containing only 0.07% phosphorus, of which less than 0.01% is phytin), or cereal mixture no. 1 containing 40 parts of corn, 20 parts of wheat middlings and 10 parts of wheat bran. Seventy-nine per cent of the total phosphorus contributed by cereal mixture no. 1 was present as phytin. When this cereal mixture comprised 67.52% of the total diet, it contributed 0.42% total phosphorus to the diet. The complete plan of the experiment appears in table 2.

Vitamin D was supplied from either a pure cod liver oil or irradiated 7-dehydrocholesterol (hereafter referred to as D_3) diluted in corn oil. The vitamin D supplements were assayed by the A.O.A.C. chick method in this laboratory and also at the Connecticut (New Haven) Agricultural Experiment Station and assigned a potency on the basis of the combined

assay data. Adequate vitamin A was furnished by the oral administration of a pure vitamin A concentrate in corn oil. The poults received the diet ad libitum. After 28 days they were weighed and sacrificed for bone ash determinations. The dry, fat-free tibia were ashed individually. Total calcium, total phosphorus and phytin phosphorus were determined for all diets. Day-old Broad Breasted Bronze turkey poults from the University of Connecticut flock were used in all experiments.

EXPERIMENTAL

In Experiment 1 two series of lots of poults were fed diets containing increasing amounts of cereal phosphorus and decreasing amounts of bone meal. A Ca : P ratio of approximately 2.5 : 1 was maintained with ground limestone. All lots were fed 80 A.O.A.C. chick units of vitamin D per 100 gm of diet, lots 1–6 receiving pure cod liver oil and lots 7–12 vitamin D_3. Thus the diets fed lots 1 and 7, 2 and 8, etc., were the same except for the source of vitamin D. It was planned to keep the total phosphorus level constant at 0.60% except for lots 6 and 12. These two lots received cereal mixture no. 2 containing more wheat by-products and less corn than mixture no. 1, thus increasing the levels of total and cereal phosphorus fed these lots by 0.17%. This was intended to demonstrate the effect, if any, on bone calcification of increasing the total phosphorus level in the diet by means of the cereal ingredients. Twenty-one poults were started in each lot. Mortality was insignificant, only three poults dying in the entire experiment. A summary of the experimental data on rations and results appears in table 2.

It is obvious from the data presented in table 2 that the per cent bone ash decreases steadily and rapidly, as the relative amount of cereal phosphorus increases. The poults of lot 5 were markedly rachitic despite the presence of 80 units of vitamin D per 100 gm of diet, and adequate levels of calcium and total phosphorus. The downward trend was similar for both sources of vitamin D, but the per cent bone ash of the poults receiving the vitamin D_3 declined less sharply and

never reached as low a point as did those receiving cod liver oil. It is interesting to note that increasing the total phosphorus level 0.17% by means of cereal phosphorus had very little effect upon bone ash when the diet contained cod liver oil (lot 6), but a marked effect when it contained vitamin D_3 (lot 12). In general, poults receiving the D_3 exhibited a bone ash value approximately equal to that of poults receiving cod liver oil and 0.10% more non-cereal phosphorus. The relationship between the level of non-cereal phosphorus and bone

TABLE 2

Summary of experimental data on rations, and results in experiment 1.

DIET VARIABLES	LOT NOS.					
	1 and 7	2 and 8	3 and 9	4 and 10	5 and 11	6 and 12
Basal diet	28.00	28.00	28.00	28.00	28.00	28.00
White corn meal	66.74	50.04	33.37	16.68	....	
Cereal mix 1.	....	16.90	33.80	50.70	67.52	67.52 [1]
Bone meal	2.86	2.15	1.43	0.72	....	....
Limestone	2.40	2.91	3.40	3.90	4.48	4.48
Totals	100.00	100.00	100.00	100.00	100.00	100.00
Analyses %						
Total Ca	1.60	1.60	1.60	1.60	1.60	1.60
Total P	0.67	0.65	0.64	0.63	0.62	0.79
Non-cereal P	0.67	0.54	0.43	0.31	0.20	0.20
Cereal P	0.00	0.11	0.21	0.32	0.42	0.59
Results						
C.L.O. (Lots 1–6) Body weight Av. 28 days (gm)	268	296	308	303	291	273
Bone ash %	44.6	43.3	40.8	37.0	33.2	34.4
Vitamin D_3 (Lots 7–12) Body weight Av. 28 days (gm)	279	308	325	321	297	304
Bone ash %	45.8	45.3	44.0	40.0	35.6	40.4

[1] Lots 6 and 12 received cereal mixture no. 2, containing 15 parts of corn, 35 parts of wheat middlings and 20 parts of wheat bran. This increased the level of total phosphorus to 0.79% and the cereal phosphorus to 0.59% for lots 6 and 12.

ash is shown graphically in figure 1. Bone ash has been plotted against the logarithm of the per cent non-cereal phosphorus in the diet and a straight line fitted to the data by the method of least squares. This line may be represented by the formula $y = 26.30 + 22.60X$, in which y represents the bone ash expected and X the logarithm of the non-cereal phosphorus,

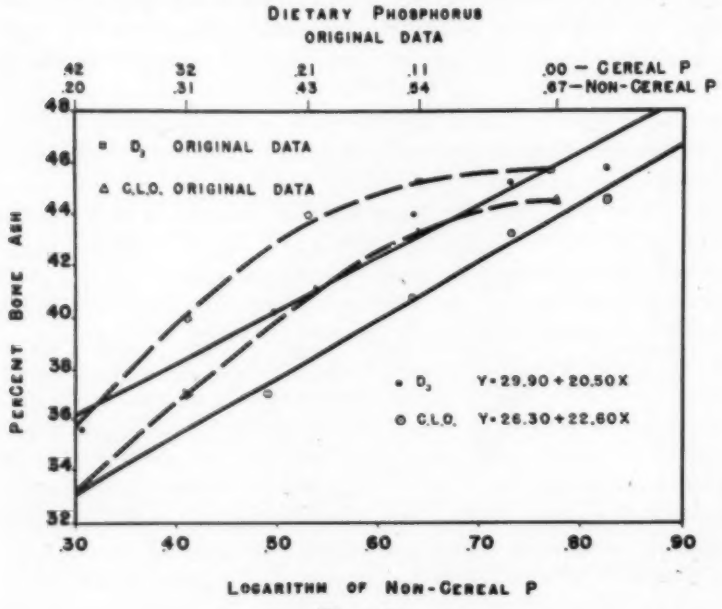

Figure 1

when cod liver oil is the source of vitamin D and $y = 29.90 + 20.50X$ when vitamin D_3 is used. The curved lines represent the original data plotted against the levels of dietary phosphorus actually fed.

In the second experiment, the diets containing non-cereal and cereal phosphorus levels of 0.31 and 0.32, and 0.20 and 0.42%, respectively, were used, since these diets were incapable of supporting normal calcification in experiment 1. Eight lots of day-old poults were placed on each diet, lots 1 to

4 receiving 60, 120, 240 and 480 units of vitamin D per 100 gm of ration, from a pure cod liver oil, and lots 5 to 8 the same unitage from vitamin D_3. The entire experiment was run in duplicate, fresh feed being mixed for each trial. The positions of the lots in the battery were selected with the aid of a randomized block based on a latin square in order to minimize the effect of position in the battery on results. Smaller numbers of poults (6 per lot) were used in this experiment owing

TABLE 3

Summary of the data on experimental rations and results in experiment 2.

LOT NO.	DIETARY PHOSPHORUS	A.O.A.C. CHICK UNITS OF VITAMIN D		RESULTS	
		C.L.O.	D_3	Body wt. at 28 days	Bone ash
				gm	%
1		60		337	37.1
2		120		325	37.2
3	Total phosphorus 0.63%	240		324	38.9
4	Non-cereal P 0.31%	480		328	39.5
5	Cereal P 0.32%		60	326	40.6
6			120	333	42.0
7			240	328	41.9
8			480	344	42.3
9		60		289	32.3
10		120		308	34.9
11	Total phosphorus 0.62%	240		323	33.5
12	Non-cereal P 0.20%	480		288	35.3
13	Cereal P 0.42%		60	286	36.2
14			120	300	38.8
15			240	314	39.1
16			480	318	39.6

to the fact that it was carried out in duplicate, and also because fewer poults were available at this time. A summary of the experimental data on rations and results appears in table 3. Since the results of the two duplicate series are very similar, they are combined for tabular presentation and statistical analysis.

The data presented in table 3 indicate that increasing the level of vitamin D in the diet will increase the bone ash, but

that the magnitude of the response is small. Increasing the vitamin D from 60 to 480 units per 100 gm of diet (lots 1 to 4) increased bone ash only 2.4% whereas in experiment 1 an increase of 0.12% of non-cereal phosphorus (compare lots 3 and 4) increased bone ash 3.8%. The situation was similar for both sources of vitamin D, although the poults receiving vitamin D_3 always showed better calcification than those receiving cod liver oil. The difference in bone ash obtained with the two sources of vitamin D was dependent, to some extent at least, on the source of the phosphorus in the diet. This can be demonstrated by studying the relationship between the level of vitamin D fed and the per cent bone ash found in the tibia. A line of best fit was calculated by the method of least squares, for the cod liver oil data obtained with each diet, and the number of units of vitamin D from cod liver oil that would be necessary to produce a bone ash equivalent to that obtained at a given level of vitamin D_3 was then computed. The data for lots 1 to 4 may be represented by the formula $y = 36.72 + .006X$ in which y is the per cent bone ash obtained and X the number of A.O.A.C. chick units of vitamin D fed. For lots 9 to 12 the formula is $y = 32.86 + .005X$. By substituting in place of y the bone ash value obtained at any given level of vitamin D_3 one may easily calculate X, the number of units of vitamin D from cod liver oil that would be required to produce that per cent of bone ash. Dividing the number of units of vitamin D required from cod liver oil by the number of units of vitamin D_3 actually fed, gives the efficacy ratio. A summary of the efficacy ratios computed in this manner for the two diets and the several levels of vitamin D used in experiment 2 appears in table 4. It is apparent that the efficacy ratios are wider in every case on the diet containing the larger proportion of cereal phosphorus. In other words as the relative amount of cereal phosphorus (largely phytin) increased, the relative efficiency of the vitamin D_3 compared with the vitamin D of cod liver oil also increased. The rapid decrease in the size of the efficacy ratio with increasing amounts of vitamin D is due to the fact, previously noted, that bone ash

responses were small for relatively large increments of vitamin D. Unless adequate levels of available minerals are present in the diet of the poult, the ability of vitamin D to promote calcification is definitely limited. One may calculate that in experiment 2, 60 units of vitamin D from cod liver oil produced 93.9% of the bone ash obtainable with 480 units and that 60 units of vitamin D_3 produced 95.8% of the bone ash obtainable with 480 units of vitamin D_3. These results certainly demonstrate that the magnitude of the improvement in bone

TABLE 4

Efficacy ratios of vitamin D from C.L.O. to vitamin D_3 on two diets varying in their source of phosphorus. Experiment 2.

A.O.A.C. UNITAGE OF D_3	DIETARY PHOSPHORUS Non-cereal P 0.31% Cereal P 0.32%	DIETARY PHOSPHORUS Non-cereal P 0.20% Cereal P 0.42%
60	10.83 : 1	13.40 : 1
120	7.38 : 1	9.67 : 1
240	3.57 : 1	5.13 : 1
480	1.93 : 1	2.77 : 1
	$y = 36.72 + .006X$	$y = 32.86 + .005X$

y = per cent bone ash; X = A.O.A.C. units of vit. D from C.L.O.

ash that may be expected when the level of vitamin D fed is increased above the minimum requirement, is quite small. Two other facts are brought out by experiment 2. First, on both diets 60 units of vitamin D_3 supported better calcification than did 480 units of vitamin D from cod liver oil, and second, 60 units of vitamin D from either source produced as good calcification in experiment 2 as 80 units of vitamin D from the same sources in experiment 1.

DISCUSSION

That the vitamin D requirements of turkeys, or of all birds and animals for that matter, are intimately associated with the levels of and the ratio between calcium and phosphorus is a generally accepted fact. It is not so well known, however, that the turkey can make little use, for bone calcification, of

much of the phosphorus contained in the cereals and cereal by-products, and that this may influence the vitamin D "requirement" just as much as the total level of this mineral. Certainly the results obtained with lots 1 and 7 in experiment 1 suggest that 80 units of vitamin D per 100 gm of ration is entirely adequate for the turkey. Yet lots 4, 5 and 11 contained several markedly rachitic birds despite the fact that the total mineral and vitamin D intake was essentially the same as that of lots 1 and 7. Increasing the vitamin D to 480 units per 100 gm of feed (expt. 2) eliminated external symptoms of rickets but increased bone ash an average of only 2.45% whereas an increase of 0.11% of non-cereal P (expt. 1, lots 4 and 5, and 10 and 11) increased bone ash 4.10%. The primary problem of bone calcification in the poult appears therefore, to be one of mineral source and level with the logical and most effective solution coming through this channel rather than through manipulation of the vitamin D level.

It has been pointed out by Matterson, Scott and Singsen ('46) and confirmed by the experiments reported here, that many of the discrepancies which appear in the literature as to the vitamin D requirement of turkey poults can be explained in part at least, on the basis of the availability of the phosphorus used in the ration. This relationship is shown clearly by plotting the per cent bone ash against the logarithm of the non-cereal phosphorus in the ration (fig. 1). When the ration contains 0.20 to 0.67% of non-cereal phosphorus, the increase in bone ash is a straight-line function of the logarithm of the non-cereal phosphorus in the diet. If we assume that an average bone ash level of 40%, although far from representing maximum storage of minerals in the bone, represents a minimum practical level of calcification which will prevent rickets, then one may easily calculate from figure 1 that the diet must contain not less than 0.306 and 0.385% non-cereal phosphorus with 80 units of vitamin D per 100 gm of feed from D_3 and cod liver oil, respectively. This assumes, of course, a total phosphorus level of approximately 0.65%. In terms of non-cereal phosphorus the difference between the two

sources of vitamin D is approximately 26% at this level of mineral intake. The percentage difference between the two sources of vitamin D decreases as the level of total non-cereal phosphorus in the diet increases. Although relatively large increases in cereal phosphorus (expt. 1, lots 6 and 12) will promote fair calcification when the diet contains vitamin D_3, this is not true when cod liver oil is used as the source of vitamin D. It is of interest to consider for a moment just what this observation implies. If approximately 75% of the additional 0.17% cereal phosphorus fed lots 6 and 12 (expt. 1) is phytin, then these lots received about 0.04% additional non-phytin phosphorus of cereal origin. While it is conceivable that this amount of available phosphorus could account for the slight increase in bone ash noted between lots 5 and 6, (1.2%) it could scarcely account for the increase noted between lots 11 and 12 (4.8%). This certainly suggests that the action of the vitamin D_3 involved the phytin as well as the non-phytin phosphorus whereas that of cod liver oil probably involved only the non-phytin phosphorus. The results obtained with cod liver oil in these experiments indicate that although small amounts of the phosphorus contributed to the diet by the cereal ingredients may be utilized for calcification, most of it cannot be used for this purpose. This finding is in agreement with the work of Matterson, Scott and Singsen ('46) who used commercial calcium-magnesium phytate, isolated from corn, as the source of dietary phosphorus. Furthermore, the trend of the difference existing between the two sources of vitamin D was similar in both series of experiments. It seems entirely reasonable to conclude, therefore, that the poor utilization of phosphorus by the poult is attributable to the inability of this species to use phytin phosphorus for bone calcification, and that the greater efficacy of vitamin D_3 as compared to cod liver oil is due in part at least to its effect upon the utilization of phytin phosphorus.

Variations in growth in both experiments are relatively small and are not related to variations in the source of phosphorus in the diet. Since it is well known that 0.20% total

phosphorus will neither support life nor growth with turkey poults, it is obvious that the cereal phosphorus, largely in the form of phytin, is being used by the body to carry on the metabolic processes necessary for growth. These findings are in agreement with those of Singsen ('45) for the chick. If decreases in growth do occur in the field, they are probably the result of low feed intake due to rickets and the resultant disinclination to move rather than to metabolic inability to grow. The fact that the birds continue to grow simply aggravates the rachitic condition.

CONCLUSIONS

On the basis of the data presented here, the following conclusions seem justified.

1. Approximately 0.65% of non-cereal phosphorus in the diet will satisfactorily support growth and bone calcification in the poult.
2. Under practical conditions 0.40% non-cereal phosphorus will support adequate calcification and maximum growth with either source of vitamin D, provided the diet contains not less than 0.65% total phosphorus.
3. The vitamin D requirement of the poult, in the presence of adequate available phosphorus and a Ca : P ratio of approximately 2.5 : 1 is not more than 80 and may be less than 60 A.O.A.C. chick units per 100 gm. of diet.
4. When the levels of calcium, vitamin D and total phosphorus are held constant, the per cent bone ash decreases with increasing amounts of cereal phosphorus.
5. The phosphorus of a mixture of corn, wheat middlings and wheat bran is relatively unavailable to the poult for bone calcification.
6. The per cent bone ash obtained is a straight line function of the logarithm of the non-cereal phosphorus when the diet contains from 0.20 to 0.67% non-cereal phosphorus, approximately 0.67% total phosphorus, and 80 units of vitamin D per 100 gm of diet.

7. Vitamin D_3 is more effective than the vitamin D from cod liver oil in promoting calcification in poults receiving a diet relatively high in cereal phosphorus and low in non-cereal phosphorus. The difference in efficacy between the two sources of vitamin D decreased as the level of non-cereal phosphorus in the diet increased.

8. The efficacy ratio between vitamin D_3 and the vitamin D of cod liver oil is conditioned by the level and source of phosphorus in the diet as well as the level of vitamin D fed.

9. Increasing the level of vitamin D eight-fold (60 to 480 units) is less effective in improving bone calcification than a 0.11% increase of the non-cereal phosphorus.

10. Cereal phosphorus will support growth which is maximum for the diet and the strain of poults used.

ACKNOWLEDGMENTS

We are indebted to Dr. John Correll of the Upjohn Company for the cod liver oil; Dr. J. Waddell of E. I. DuPont de Nemours and Company for the activated 7-dehydrocholesterol and to Dr. P. L. Harris of the Distillation Products Company for the vitamin A distillate.

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SOME EFFECTS OF DIETARY ZINC DEFICIENCY IN THE MOUSE[1]

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THREE FIGURES

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Attempts to produce a deficiency state in mice by means of diets deficient in zinc have been made by Bertrand and Benzon ('22), McHargue ('26), Hubbell and Mendel ('27) and Bertrand and Bhattacherjee ('35). These experiments were unsuccessful either because the diets were not low enough in zinc or they were incomplete in other respects.

Several investigations based on the use of rats have been successful in the production of an unquestionable zinc-deficiency state, as summarized in a recent review (Hegsted, McKibbin and Drinker, '45). If the diet is extremely deficient in zinc growth is markedly impaired (Hove, Elvehjem and Hart, '38); certain specific pathological changes occur (Follis, Day and McCollum, '41) and one enzyme system, the phosphatases, is affected (Hegsted et al., '45).

Although it appears probable that no primary differences occur between rats and mice in respect to the nutritional role of zinc, it is desirable to have direct experimental evidence

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concerning both species. In addition, mice require relatively little food and space.

A logical procedure in attempting to elucidate the nutritional role of zinc is to systematically test the activity of different enzyme systems and hormonal functions in zinc-deficient animals. Schultze and Kuiken ('41) found that catalase, which is an iron porphyrin complex, is much decreased in activity by feeding a copper-deficient diet. In addition, Coulter and Stone ('38) and Kapp ('39) have each reported the isolation of a zinc porphyrin compound from biological material. Therefore, in addition to certain other observations, it was of interest to determine whether a deficiency of zinc would affect the catalase activity of selected tissues.

METHODS

Diet

The preparation and composition of the zinc-deficient diet was the same as described by Day and McCollum ('40) in studies with rats except that the level of the purified casein hydrolysate was 18% and no egg white was used. Owing to the difficulties of determining zinc quantitatively when the concentration is exceedingly low it can only be estimated that the diet contained not more than 0.3 parts per million of zinc, as determined by the Caughey, Holland and Ritchie ('38) method.

The nutritional adequacy of the diet, when supplemented with zinc, was proved by its ability to promote good growth and well being over long periods of time.

Care of the animals

Mice of the CHI strain [2] were used throughout. Individual litters, with their mothers, were removed from the stock breeder cages when they were 16–18 days old and placed in zinc-free cages containing acid-washed filter paper. The zinc-

[2] The breeding stock was obtained from Dr. L. C. Strong of the Yale University School of Medicine.

low diet was furnished. After 7 to 10 days the mother was returned to the stock colony and the litter was divided as equally as possible with regard to weight and sex.

The cages consisted of 10-liter glazed stone jars each containing a 3-mesh monel metal floor raised 1 inch above the bottom of the jar. Small pyrex glass food cups and drinking tubes were used. The water was distilled in pyrex glass. The cages were washed frequently and rinsed with hot zinc-free water. Contamination of the animals by dust and other possible extraneous sources of zinc was avoided as much as possible by keeping them in a room reserved for that purpose.

Zinc, as zinc sulfate, was given to the control animals each day by pipetting 0.2 ml of an aqueous solution onto the food. This furnished 40 μg of zinc per mouse.

Determination of catalase activity

Zinc-deficient mice and their controls were lightly anaesthetized by ether and decapitated. Two 0.1 ml aliquots of the shed blood were each pipetted immediately into 10.0 ml of redistilled water. These stock dilutions were further diluted immediately before making the catalase determinations.

The liver and kidneys were removed immediately, rinsed, blotted with ash-free paper, weighed, and ground to a thin paste in a zinc-free mortar. Then each tissue was diluted so that 1 ml contained 10 mg of tissue. After being refrigerated for about 10 hours the samples were centrifuged and the supernatant liquids were used for catalase determinations. The kidney supernatant was used without further dilution but the liver was diluted again, 4 ml of liver supernatant to 6 ml of water.

Catalase determinations were made in accordance with the method of Schultze and Kuiken ('41) except that the reaction interval was 6 minutes instead of 2. The catalase activity of liver and kidney was expressed in terms of the nitrogen content of the samples analyzed, instead of the dry weight. Greenstein et al. ('42) and some others have followed this

practice. Therefore, all the diluted liver and kidney samples were analyzed for total nitrogen, using a conventional micro-Kjeldahl method.

RESULTS

Growth and survival

About 170 mice were used in experiments which resulted in significant growth data. Impairment of growth in young mice on the zinc-deficient diet became manifest within 2 weeks. After 5 to 7 weeks surviving animals had practically ceased to grow. This was usually followed by loss of weight and eventual death. Figure 1 illustrates the effects on growth.

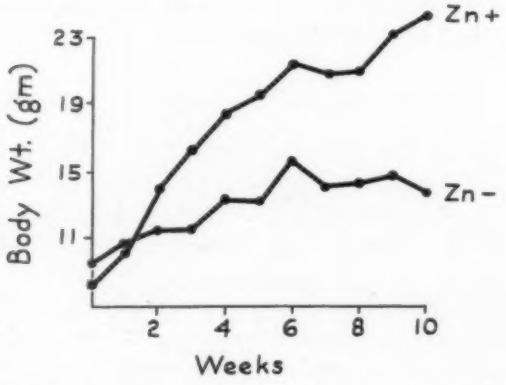

Fig. 1 Effect of dietary zinc deficiency upon the growth of young male mice. The growth of females is qualitatively the same.

Approximately 18% of the animals on the zinc-deficient diet failed to survive more than 8 weeks. This figure is based on the records from 125 mice. The survival of controls (45 animals) was 100%. Most of the fatalities occurred between the third and fifth weeks.

Within the first 2 to 3 weeks the zinc-deficient mice showed signs of progressive emaciation; the coat became greasy and unkempt; and loss of hair from the top of the head, neck and shoulders occurred in approximately 50%. Whether the

alopecia was directly related to the zinc deficiency is regarded as questionable. It may be significant that the affected parts were only those that can not be licked by the animals. The general appearance of the mice is illustrated in figure 2. Supplementation of the diet with inositol (0.5 mg per mouse per day) did not affect the alopecia.

Analyses of the bones (femurs and tibia-fibulas) by standard methods (Day and Follis, '41) showed that the percentage

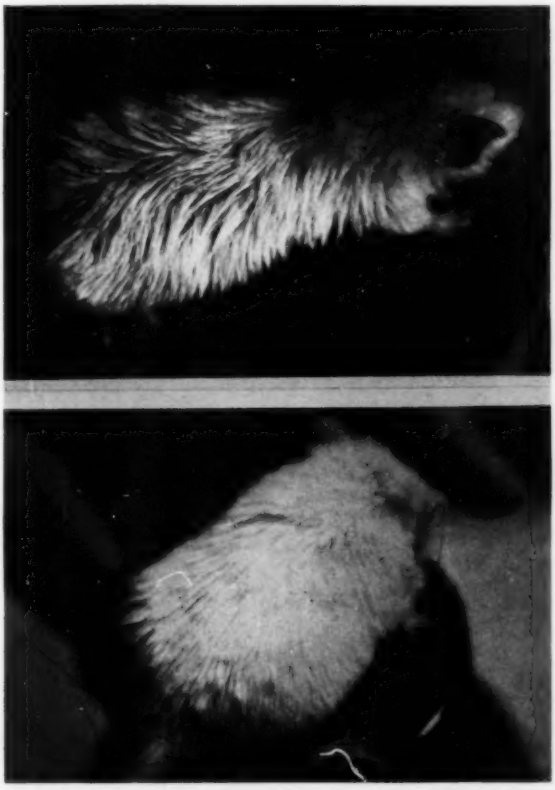

Fig. 2 The two male mice are littermates. They were on the zinc-deficient diet 28 days, but the lower one received 40 μg of zinc per day added to the food as zinc sulfate.

of bone ash was not changed by zinc deficiency although the total weight of ash was markedly reduced, as shown in table 1.

Also, the growth of the liver and kidneys was correspondingly decreased by the deficiency (table 1). All the evidence indicated that the lack of zinc had a generalized effect on growth and that the element probably is essential in many, if not all, structures of the body.

TABLE 1

Effect of zinc deficiency on the mineralization of bone and the growth of liver and kidneys.

NO. MICE AND SEX	DAYS ON DIET	LIVER	KIDNEY	BONE [1]	
				Ash	% Ash
		gm.	*gm.*	*mg.*	
		Zinc-deficient			
10 M	30	0.69	0.16	37.8	50.2 ± 2.3 [2]
6 F	31	0.74	0.17	38.5	52.2 ± 4.1
		Zinc-added			
9 M	30	1.27	0.26	47.5	48.9 ± 2.7
5 F	31	1.30	0.22	45.3	48.9 ± 4.8

[1] Femurs, tibias and fibulas combined.

[2] Average deviation of a single determination from the arithmetical mean.

Effects of cadmium

Owing to the close chemical similarities between zinc and cadmium tests were made to determine the effects of the latter on the growth of zinc-deficient mice. The dosage of cadmium, as cadmium sulfate, was 4 μg per mouse per day. The element was without ability to substitute for zinc, as indicated by the growth data from 21 animals.

Catalase

Table 2 summarizes the results of the catalase determinations. The catalase activity of liver and kidney was markedly decreased in both males and females on the zinc-deficient diet, as shown by comparison with controls given the same diet with zinc added and by comparison with others on a stock

diet. This difference was substantially the same whether expressed in terms of tissue nitrogen, dry weight or wet weight. The blood catalase activity was not changed.

In a number of cases the effect of added zinc, as zinc sulfate, was determined. The results, given in figure 3, represent the

TABLE 2

Effects of zinc deficiency on the catalase activity of mouse liver, kidneys and blood.

DIET	NO. MICE AND SEX	DAYS ON DIET	WT. GAIN WHILE ON DIET	LIVER	KIDNEYS	BLOOD
				ml 0.02 N H_2O_2 decomposed per		
				mg N	mg N	ml blood
			gm			
Zn—	14 M	27	1.7	95	129	3037
Zn+	15 M	27	9.8	187	174	3133
Stock	4 M	..	..	170	178	3247
Zn—	10 F	26	1.8	77	74	2916
Zn+	4 F	26	10.5	149	90	2790
Stock	3 F	..	..	140	120	3178

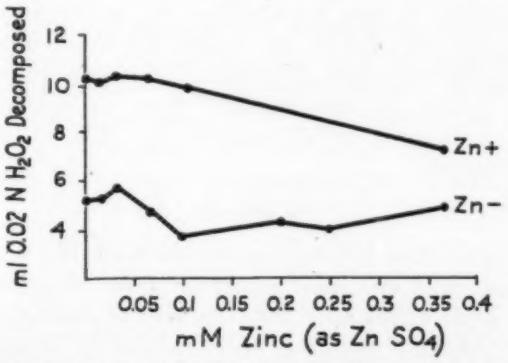

Fig. 3 The effect of zinc, added as zinc sulfate, on the catalase activity of liver from zinc-deficient mice and from controls receiving zinc in the diet.

general findings when liver is used. There was no appreciable change in the liver catalase activity either in zinc-deficient or control mice when zinc sulfate was added in amounts ranging from 0 to 0.37 mm of zinc. Considerably larger concentrations of zinc caused a slight decrease in the activity.

The large sexual difference with respect to the catalase activity of liver and kidney, shown in table 2, confirms the results reported by Schultze and Kuiken ('41). The values were highest in the males.

Liver esterase

No changes were found in the activity of liver esterase, as determined by the method of Harrer and King ('41). The average value for liver from zinc-deficient mice was 0.81, expressed as ml of 0.01 N alkali per mg of dry tissue, per 10 minutes. The average for control animals was 0.77. The activity of this enzyme decreases in ascorbic acid deficiency (Harrar and King, '41) and in some other deficiency states.

Riboflavin [3]

Owing to corneal changes which appeared to simulate those seen in riboflavin deficiency (Follis et al., '41) it was believed that a deficiency of zinc might affect the metabolism of riboflavin and that changes in the concentration of this vitamin in the tissues might occur. However, the riboflavin content of liver and kidney of zinc-deficient mice, as determined by microbiological assay (Snell and Strong, '39) was the same as in the controls.

Teeth [4]

Histological examinations were made of the teeth from zinc-deficient mice and their controls.[5] Over 20 animals were examined. Four of the mice had been on the zinc-deficient diet 89 days. Although weanling mice invariably failed to survive that long on the diet these 4 were exceptions. They were 37 days old before being restricted to the zinc-deficient diet.

[3] The riboflavin determinations were made by Dr. Kenneth P. Broshears and Dr. L. S. McClung to whom we are indebted.

[4] The authors deeply appreciate the help Dr. Schour rendered in making this part of the investigation possible.

[5] This was done by Dr. Isaac Schour of the University of Illinois College of Dentistry. The animals were killed at Bloomington and preserved in formalin before being sent to Dr. Schour.

No changes in enamel or dentin were found in either the incisors or molars. The average length of time on the experimental diets was approximately 47 days. All mice deprived of zinc were extremely deficient as indicated by their appearance, poor growth, and the catalase activity of their livers and kidneys.

DISCUSSION

The observations recorded here, in addition to those elsewhere concerning the effects of zinc deficiency upon the rat (Hegsted et al., '45) demonstrate the essentiality of zinc for higher animals. The results show liver and kidney catalase must now be added to the very small list of enzyme systems known to be affected by this deficiency. However, the investigations to date have not revealed any biochemical lesions or pathological effects of such magnitude that they would seem to be fully accountable for the baneful effects of extreme zinc deficiency.

Debilitating conditions in general do not all affect the catalase activity of tissues. Our unpublished results with rats made extremely deficient in folic acid by the addition of succinylsulfathiazole to a purified diet has shown that the catalase activity of liver, kidney and blood is not decreased. Lepkovsky and Parsons ('43) found no decrease in the activity of this enzyme in pyridoxine-deficient rats. Also, starvation up to 3 days does not affect liver catalase activity (Greenstein et al., '42).

Some instances of marked differences between tissues with respect to the degree of change in catalase activity may be found in the literature. Schultze and Kuiken ('41) reported that the catalase of blood, as well as that of liver and kidney, underwent a marked decrease in activity when the animals were deficient in either copper or iron. However, the activity of catalase in the ventricles was increased in copper deficiency. Greenstein et al. ('42) observed no decrease in blood catalase activity of tumor-bearing mice and rats even though the activity of the enzyme in the liver and kidneys was greatly decreased.

The function of zinc in affecting catalase must be regarded as indirect because the addition of zinc salts to tissue preparations from zinc-deficient mice does not increase the catalase activity. Further evidence in this direction is the fact that none of the crystalline catalase enzymes investigated have been found to contain zinc.

No evidence is available to explain the decrease in catalase activity. It might be due to a general decrease in the rate of production; but if this were true the activity of blood catalase should also decline. Another consideration is that the catalase in liver and kidney is not the same as that in blood. Some support of this hypothesis is afforded by Sumner, Dounce and Frampton's ('40) discussion of the possible occurrence of several catalases differing in activity which, it is suggested, might "result from enzyme action in the liver (or kidneys) of an animal before or after death." On this basis it is possible that the catalase in the liver and kidneys of the zinc-deficient mice consisted of less active enzyme components than the catalase in corresponding tissues of the control animals. If sufficient quantities of tissue material from zinc-deficient animals could be secured it would be interesting to test this hypothesis by attempting to concentrate the enzyme(s) and compare the "Kat.f." of such catalase with that from control animals.

SUMMARY

Weanling mice on an extremely zinc-deficient diet were greatly retarded in the rate of growth. A considerable percentage failed to survive more than 8 weeks. The animals became emaciated, lost hair from the shoulders and back of the neck, but no truly distinctive gross symptoms were noted. This is regarded as evidence that in nutrition zinc has diverse functions.

The catalase activity of the liver and kidneys was markedly reduced. Blood catalase was not affected. The addition of zinc salts to the tissue preparations did not appreciably affect the catalase activity.

No changes occurred in liver esterase and in the concentration of riboflavin in liver and kidneys.

Histological investigations of the teeth failed to reveal any evidence of change attributable to the lack of zinc.

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SEASONAL VARIATIONS IN THE VITAMIN A AND THE CAROTENE CONTENT OF RETAIL BUTTERS[1]

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THREE FIGURES

(Received for publication August 27, 1946)

Early in 1941 the Office of Experiment Stations in Washington, D. C., invited the State Agricultural Experiment Stations to cooperate in a nation-wide study relative to the carotene and the vitamin A content of butters produced throughout the country. To date, reports have been published by the following collaborating laboratories: Wisconsin (Berl and Peterson, '43); Minnesota (Jenness and Palmer, '45); U. S. Dept. Agric., '45; and Kansas (Parrish, Martin, Atkeson and Hughes, '46).

The study described in the present paper was a part of the national cooperative project and was designed to yield information regarding seasonal fluctuations in carotene and in vitamin A in typical market butters as purchased on the Pennsylvania retail market.

EXPERIMENTAL

Collection of samples

The butters used in this study consisted of 5 nationally-advertized brands and 1 brand produced by a local

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creamery. The nationally-advertized butters were purchased on the retail market while the product of the local creamery was purchased directly from the creamery. In general, the samples were purchased semi-monthly. Whenever possible, 1 sample of each product was purchased in the first half and the second sample in the last half of each month. Because of the limited available supply of butter during certain portions of the period of investigation, it was not always possible to obtain 2 samples of each brand of butter in a given month. To make the study more complete, samples of the respective butters which were not available during the current year were purchased during the corresponding weeks and months of the following year. The investigation, therefore, extended for 2 years, from October 1943 to October 1945.

All samples of butter were scored for quality by representatives of the Division of Dairy Manufacturing previous to being assayed. The butters, on being received at the laboratory, were allowed to stand at room temperature until sufficiently warm to permit thorough mixing. The mixing was conducted in a porcelain evaporating dish by means of a porcelain spatula. The butter samples were then placed in airtight glass jars and stored in a refrigerator at 5°C. until they could be assayed.

Methods of analysis

All samples were analyzed for vitamin A and for carotene by the procedures described by Koehn and Sherman ('40) after certain modifications as recommended by the Technical Committee appointed for the National Project by the Agricultural Research Administration. These methods have been outlined in considerable detail by Jenness and Palmer ('45). Since the sources of some of the butter samples were unknown, 92% methanol was used throughout the investigation to extract the noncarotene pigments. Light transmission measurements were made by means of an Evelyn photoelectric colorimeter, using a 620 mμ filter for vitamin A measurements

and a 440 mμ filter for establishing the vitamin A correction factor and for beta carotene measurements. Concentrations of vitamin A and carotene were determined by use of standard reference curves prepared with crystalline vitamin A alcohol and with crystalline beta carotene.

DATA

The results of this investigation are presented in condensed form in figures 1–3, inclusive.

DISCUSSION

Methods of analysis

While carotene and vitamin A were determined by the assay procedures indicated above, certain tests were made during the course of the investigation for the purpose of ascertaining the possible effects of certain variations in laboratory procedures, reagents, etc., on the results of the assay. The results of some of these observations appear to merit mention.

Reports have appeared in the literature relative to the merits of 92% methanol or of 94% diacetone alcohol in the phasic separation and the use of a chromatographic procedure for separating the noncarotene pigments from beta carotene when the carotene is dissolved in petroleum ether or in Skellysolve (Berl and Peterson, '43; Hauge, Westfall, Wilbur and Hilton, '44; and Zscheile, Nash, Henry and Green, '44). Investigations carried out along this line, using a limited number of samples of butter, revealed that when the solutions were extracted with 94% diacetone alcohol approximately 11% less pigment remained than when the extraction was made with 92% methanol (for example, 4.71 μg of carotene per gm as against 5.31 μg per gm). Furthermore, in the chromatographic procedure, where dicalcium phosphate was used as the adsorbing agent, 10% less pigment remained than when the extraction was made with 92% methanol (for example, 5.56 μg per gm against 6.17 μg per gm). While it cannot be said that these findings will apply to all types of butters,

these results are in general agreement with those reported by the above investigators.

Some observations were made regarding the effect of using antimony trichloride reagent of variable degrees of freshness. In the studies herein reported the reagent was always stored in a brown, glass-stoppered bottle in a dark compartment of the laboratory desk. After several months of storage, it was found that the reagent yielded results comparable with those obtained with the freshly-prepared reagent. This is contrary to the findings of Edisbury ('40) but agrees with the findings of Dann and Evelyn ('38) and those of Benham ('44). In fact, it was observed that when the residual solution and undissolved crystals from several bottles of the reagent were combined in 1 bottle over a period of months, the combined reagents yielded vitamin values which agreed with those obtained when the freshly-prepared reagent was employed. However, as pointed out by Dann and Evelyn ('38), when the older reagent was used the blue color was observed to fade more rapidly.

Peroxides, frequently present in diethyl ether, were found to have a marked deleterious effect on vitamin A but appeared to have no significant effect on carotene. This is illustrated by the data presented in table 1.

TABLE 1

Effect of using diethyl ether containing traces of peroxides in the analysis of butters for vitamin A and carotene.

SAMPLE NUMBER	CONCENTRATION (μG PER GM)			
	Vitamin A		Beta carotene	
	Peroxide-containing ether	Peroxide-free ether	Peroxide-containing ether	Peroxide-free ether
126	6.22	9.82	5.12	5.12
127	4.16	7.50	6.17	6.17
128	4.62	7.44	6.23	6.44
129	6.34	9.40	6 87	6.70
130	7.32	9.48	6.17	5.97
131	6.22	8.94	7.92	7.85
133	5.38	9.74	4.74	4.89

Investigations carried out for the purpose of determining the per cent recovery of vitamin A alcohol and beta carotene, when added to butter, gave somewhat varying results, but in general indicated that the losses were negligible. While small losses of added vitamin A have been reported by other investigators (Zscheile, Henry, White, Nash, Shrewsbury and

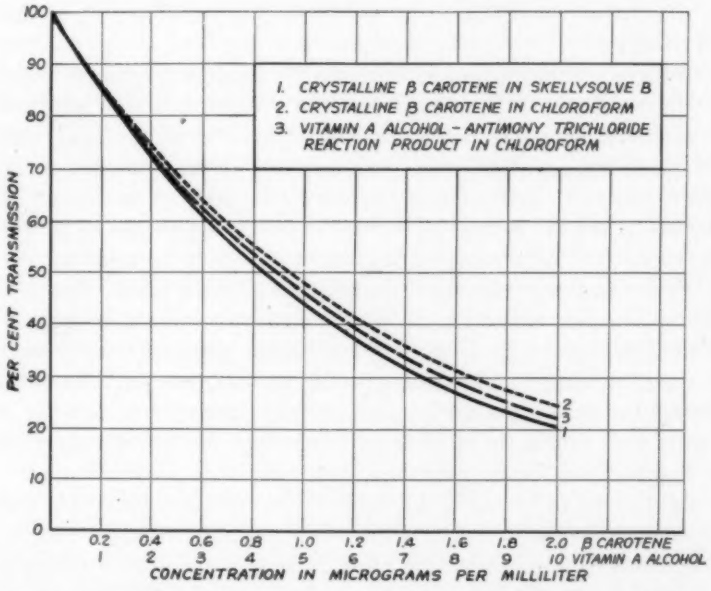

Fig. 1 Concentration curves used in evaluating the carotene and the vitamin A content of butters. The concentration of beta carotene was measured while using a 440 mμ filter and that of vitamin A-antimony trichloride reaction product with a 620 mμ filter.

Hauge, '44; and Jenness and Palmer, '45), the results reported in this publication have not been corrected for loss of vitamin during analysis inasmuch as no appreciable loss was detected.

The standard curves used in evaluating the vitamin values reported at this time are given in figure 1. The K values for

these curves when calculated between 30 and 80% transmission were 14.86 for vitamin A alcohol-antimony trichloride reaction product, 2.77 for beta carotene in Skellysolve B, and 3.15 for beta carotene in chloroform, where K equals concentration in micrograms per milliliter divided by 2–log G (Galvanometer reading). Slightly different standard curves were used in evaluating the potencies of butters reported in a previous publication (U. S. Dept. Agric. Misc. Pub. No. 571, '45).

Before evaluating accurately the probable nutritional value of butter from its reported vitamin A and carotene content, one must recognize the following sources of error: (a) lack of complete separation of beta carotene from contaminating pigments, (b) lack of a satisfactory vitamin A standard in terms of which biological values may be expressed, and (c) lack of definite conversion factors which may be used to convert physical measurement data into biological units. Some of the difficulties encountered in attempts to separate beta carotene quantitatively from contaminating pigments by phasic separation and by chromatographic procedures have been indicated above. In addition to existing limitations, neither of these procedures is expected to separate quantitatively beta carotene from alpha carotene, cryptoxanthin and other carotenoids having lower biological values. Gridgeman ('44) has reviewed much of the published literature relative to the variability of the proposed vitamin A and carotene standards as well as that relating to some of the suggested conversion factors. The authors have determined the extinction coefficients of numerous samples of what was supposed to be pure crystalline vitamin A alcohol and pure crystalline beta carotene as purchased from supply houses. The results of these measurements showed $E\,\frac{1\%}{1\,cm}$ at 328 mμ ranging from 1320 to 1990 for vitamin A alcohol and $E\,\frac{1\%}{1\,cm}$ at 450 mμ from 2000 to 2590 for beta carotene. The differences in the extinction coefficients of the contents of vials having the same control number was invariably less than the differences between vials having different control numbers.

The vitamin A values herein reported have been corrected for the presence of carotene in the test solution by applying the correction factor 0.007 as directed by the Technical Committee on Methods for this general project. However, when solutions of beta carotene in chloroform, ranging in concentration from 10 to 22 μg per ml, were prepared and the effect of the antimony trichloride reagent tested as suggested, the results indicated a correction factor of 0.004. Berl and Peterson ('43) had previously reported the correction factor to be 0.007, while Parrish, Martin, Atkeson and Hughes ('46) have more recently reported this factor to be 0.005. However, any correction factor determined as indicated above does not take into consideration any $SbCl_3$-reaction product that may be formed by some of the pigments other than beta carotene known to be present in butter. Furthermore, the presence of different amounts of these pigments in butter would be expected to lead to correction factors different from those indicated above. The authors are of the opinion that errors in assay values arising from these sources are also worthy of consideration.

Results of butter analysis

The seasonal variations in the vitamin A and carotene content of butters purchased on the local retail market, as demonstrated by the examination of 142 samples over a period of 2 years, are shown by the data presented in figure 2. In this figure, the five nationally-advertized butters are indicated by numbers 1–5, inclusive, while the butter from the local creamery is designated as butter number 6. An examination of these data as they are related to butter score indicated that there was no apparent relationship between the usual quality score and the vitamin content of the respective butters. These observations are in agreement with those of Berl and Peterson ('43) and also those of Parrish, Martin, Atkeson and Hughes ('46).

Some interesting generalizations concerning the relationship and the variability of vitamin A and carotene in the

nationally-advertized butters may be drawn as the result of further examination of the data presented in figure 2. It is found that the average vitamin A content of the 5 butters during the months of January–June, inclusive, was 0.526 mg

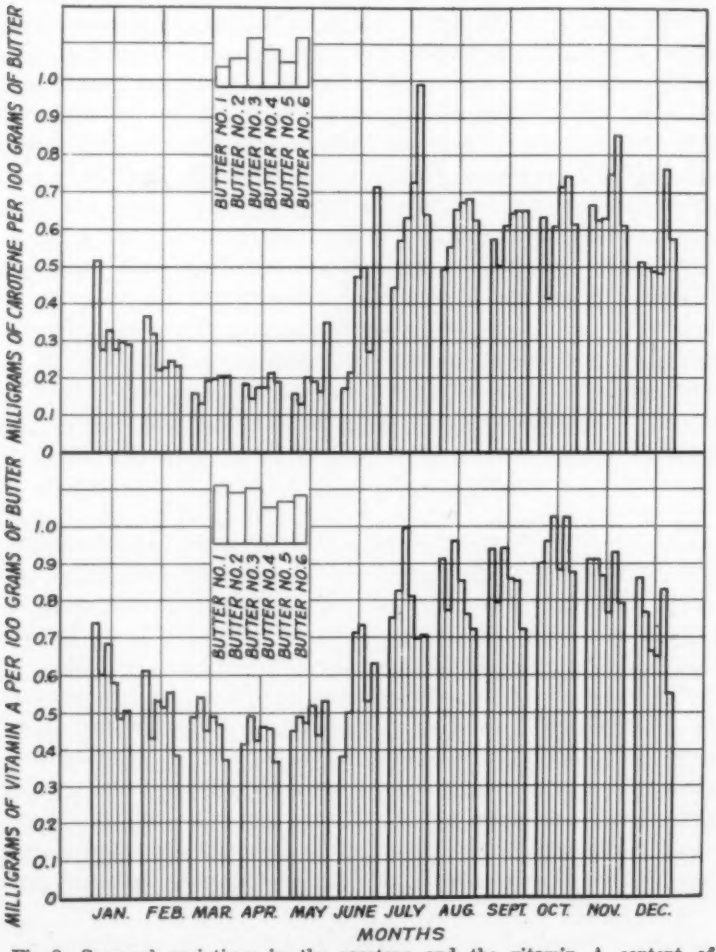

Fig. 2 Seasonal variations in the carotene and the vitamin A content of butters as purchased on the retail market.

per 100 gm of butter while the average vitamin A content of the same butters during the months of July–December, inclusive, was 0.857 mg per 100 gm. Accordingly, "summer–fall" butters contained an average of 63% more vitamin A than "winter–spring" butters. Likewise, it is found that the average carotene content of the 5 nationally-advertized butters during the months of January–June, inclusive, was 0.244 mg per 100 gm of butter, whereas the average carotene content of the same butters for the remaining 6 months of the year was 0.625 mg per 100 gm. These "summer–fall" butters therefore contained an average of 165% more carotene than the corresponding "winter–spring" butters. When one considers 1 μg of vitamin A equivalent to 4 I.U. and 1 μg of the above carotene equal to 1.67 I.U., these data reveal that the average vitamin A potency of these butters exceeded the carotene equivalent during the months of January–June, inclusive, by 416%, whereas it only exceeded the carotene equivalent by 228% during the remaining months of the year. In addition, the vitamin A content of these butters remained somewhat more constant throughout the year than did the carotene content. This is in agreement with the findings of others (Berl and Peterson, '43; Jenness and Palmer, '45; Lord, '45; and Parrish, Martin, Atkeson and Hughes, '46). As would be expected, similar observations have been reported for vitamin A in bovine blood plasma (Sutton and Soldner, '45; and Lord, '45).

While the data show that there were measurable differences in the vitamin A and in the carotene content of different butters during a given month, the average vitamin values for the respective months indicate a definite seasonal variation in vitamin content. Intraseasonal variations in vitamin A and carotene content have been reported by Berl and Peterson ('43), Jenness and Palmer ('45), and Parrish, Martin, Atkeson and Hughes ('46). In the present studies, however, there were no indications that any one butter was continually higher or continually lower in either vitamin A or carotene than other butters under investigation.

The data presented in figure 3 are based on the previous assumption that one International Unit of vitamin A is equivalent to the biological effect of 0.25 μg of vitamin A alcohol (Berl and Peterson, '43) or to 0.6 μg of beta carotene (Quart. Bull. Health Organization, League of Nations 4, 431,

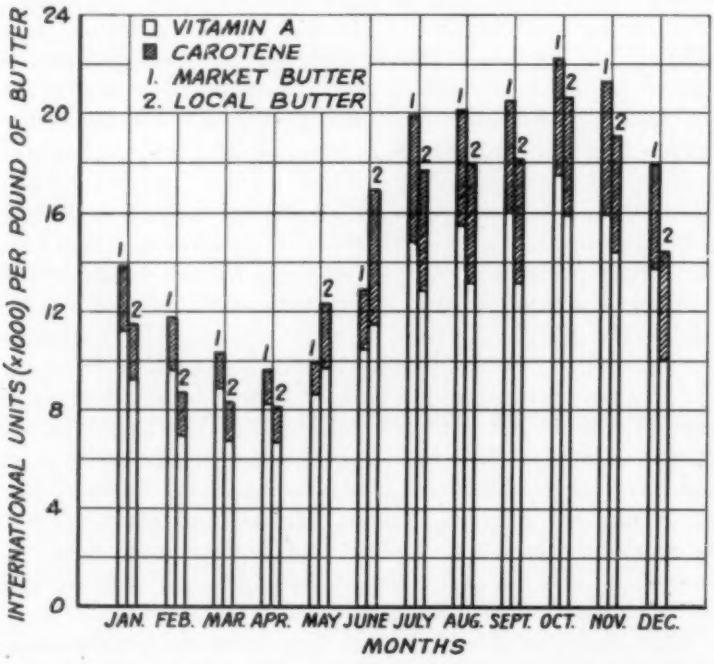

Fig. 3 Seasonal variations in the contribution that carotene and vitamin A made to the biological potency of butters. The values given for the market butter are the average values obtained for the 5 nationally advertized butters.

1934). However, biological assays conducted in this laboratory do not substantiate this relationship for vitamin A alcohol, the alcohol being somewhat less potent than the above figure would indicate. The butter purchased from the local creamery had the lowest vitamin potency during the months of February, March and April with the potency showing a

rapid rise during May and June, with further but more gradual increases in potency during July, August and September. The highest potency occurred in October. The 5 commercial butters showed the lowest potency during the months of March, April and May with the potency rising rapidly during June and July, a tapering off in the rate of increase during August and September, with the highest potency likewise occurring in October. Perhaps it should be expected that these butters would reach their peak in vitamin potency in November, considering the delay observed in the rate of vitamin enrichment during the spring months. Although the data show that the biological potencies of the butters increased during the months of June, July, August and September, with the highest potency occurring in October, the data also reveal that the amount of potency attributable to carotene was somewhat less during August and September than during either July or October. Perhaps this reflects the carotene content of the dairy ration.

The average vitamin A potency of the 6 brands of butter for the year was found to be 15,640 I.U. per pound. Considering the possible differences in standards used, this value compares favorably with those reported in the literature. Of this potency, 12,290 I.U. were calculated as having been contributed by vitamin A, while 3,350 I.U. were contributed by carotene.

The data regarding seasonal variation in the vitamin A potency of the butter purchased at the local creamery are in agreement with the data reported elsewhere concerning similar butters (Jenness and Palmer, '45; Lord, '45; and Parrish, Martin, Atkeson and Hughes, '46). Inasmuch as the over-all seasonal variation in the vitamin potency of the commercial butters paralleled the variations in the vitamin potency of the fresh butter obtained from the local creamery, these data indicate that, during the period of this study, commercial butters moved from manufacturer to consumer without delay. When possible differences in the vitamin standards used by different investigators are taken into consideration, these data further reveal that the total vitamin A potencies

of the 5 commercial butters herein reported compare favorably with those values reported for butter taken at the point of manufacture. This would tend to show that no significant loss in vitamin potency takes place during the normal period of time required for butter to pass from the manufacturer to the consumer. The present findings regarding this matter are in full agreement with the summarized results thus far reported by collaborators participating in the national butter project.

SUMMARY

A study of the seasonal variations in the vitamin A and carotene of commercial butter as purchased on the Pennsylvania market is described. In the course of a 2-year period more than 140 samples of butter were assayed. These samples represented 5 nationally-advertized brands of butter purchased on the retail market and 1 brand purchased at a local creamery.

Preliminary to and during the course of these assays, studies relating to methodology were carried out for the purpose of checking or improving methods of assay. The results of these studies are summarized as follows:

1. Freshly-prepared solutions of the antimony trichloride reagent and solutions which had been prepared for several months yielded essentially the same vitamin A values when used with the same butters.

2. In the analysis of butter, the 94% diacetone alcohol procedure and the chromatographic procedure for eliminating the noncarotene pigments yielded lower carotene values than did the procedure in which 92% methanol was used.

3. Peroxides, frequently found in diethyl ether, were found to exert a marked deleterious effect on vitamin A stability but appeared to have no effect on carotene stability during the usual course of the assay.

4. Some of the vitamin A standards and carotene standards in use at the present time were found to vary with respect to their extinction coefficients.

5. Although distinct seasonal variations in the carotene and vitamin A content of butters as purchased on the retail market in Pennsylvania were noted, there was no indication that any one brand of butter was constantly higher or constantly lower than other brands with respect to either vitamin A or carotene.

6. With few exceptions, the vitamin A content of butter was found to exceed the carotene content (in terms of both weight of material present and International Unit equivalent) at all seasons of the year and also showed less seasonal variation.

7. The average yearly vitamin potency of the 6 brands of butter was found to be 15,640 I.U. per pound.

8. A comparison of the seasonal vitamin A potencies of the commercial butters with those of the butter from the local creamery and with vitamin values reported in the literature for fresh butters indicated that during the course of this study the vitamin content of butter was relatively stable and that not much time elapsed from the date of manufacture until the butter reached the consumer.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Professor I. E. Parkin of the Division of Dairy Husbandry Extension for collecting the samples of butter used in the early part of the investigation and to Professors F. J. Doan and D. V. Josephson of the Division of Dairy Manufacturing for scoring the numerous samples of butter used throughout the investigation.

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ABSORPTION AND STORAGE OF VITAMIN A IN THE LIVER OF THE RAT

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It has long been recognized that the animal body is capable of accumulating vitamin A and that the liver is the chief storage site (Osborne and Mendel, '18). Studies regarding the distribution of stored vitamin A in the rat's body have shown that 90 to 95% is found in the liver (Sherman and Boynton, '25; Moore, '31; McCoord and Luce-Clausen, '34) and that the liver content of the vitamin is usually proportional to the intake (Baumann, Riising and Steenbock, '34). The liver stores of the vitamin may indicate both the state of nutrition of the animal and the availability of the vitamin when administered under different conditions. In the following pages an investigation of the influence of the sex of the animal, the level of intake, the source of the vitamin and the mode of administration on the storage of vitamin A in the liver of the rat is reported.

PROCEDURE

Weanling rats from our breeding colony were maintained in air conditioned quarters (75°F., 40% relative humidity) on the U.S.P. vitamin A-free diet composed of the following ingredients: purified casein, 18%; acetone-washed cornstarch, 65%; salt mixture, 4% (Jones and Foster, '42); acetone washed brewer's yeast, 8%; cottonseed oil, 5%; vitamin D, 6 units per gm of ration. When their weights remained stationary or declined for a period of 7 days they were considered to be depleted; this required from 25 to 35 days. They were

then divided into comparable groups of 6 and treated for 3 days with daily supplements of vitamin A. The supplements were administered orally by pipette unless otherwise stated. A short term treatment period was adopted in accordance with the observations that this method had given results which were as accurate as long term experiments (Sherman and Todhunter, '34; Guggenheim and Koch, '44).

In these studies on liver storage the daily level of vitamin A ranged from 250 to 1000 International Units (I.U.) per rat. This range was arbitrarily chosen since it had been found in preliminary work that the minimum doses required for normal growth did not promote any liver storage of the vitamin. To promote a rapid accumulation of vitamin A in the liver it was necessary to administer much more than the minimum required dose.

After the 3-day treatment the animals were rested for 1 day and on the fifth day were killed by decapitation. The livers from each group of rats were weighed, pooled, homogenized in a Waring blendor and samples taken for determination of vitamin A. In our early work determinations were made on individual livers but the small variations obtained did not justify this procedure.

Two to 5 gm samples of liver tissue were weighed and mixed with 60% KOH (0.5 ml/gm liver) and 95% alcohol (5.0 ml/gm liver). The mixture was heated on a steam bath until saponification was complete. The samples were then transferred to separatory funnels, water was added (10.0 ml/gm liver) and the tissue extracted twice with 50 ml portions of peroxide-free ethyl ether. Ethyl ether was used because Benham ('44) had reported that it gave more complete extraction of vitamin A than petroleum ether. The ether extract was washed thoroughly with distilled water until the wash water gave no color with phenolphthalein. After washing, the extract was dried by filtering through anhydrous Na_2SO_4, the ether removed by vacuum distillation and the residue dissolved in chloroform. The vitamin A content of the solution was determined by the Carr-Price reaction (Carr

and Price, '26) using the Evelyn colorimeter with filter no. 620. One ml of the chloroform solution was dispensed into a standardized colorimeter tube, the tube placed in the colorimeter and 9 ml of a 22% solution of antimony trichloride in chloroform were added rapidly. A reading was taken at the first momentary stop of the galvanometer needle and the "L" value, or optical density, corresponding to the galvanometer reading was calculated ($L = 2 - \log G$). This result was then converted to International Units of vitamin A using a reference curve. A vitamin A distillate (no. 78794),[1] which contained 401,000 I.U. per gm was employed as a standard in preparing the above curve.

Both the preparations used as supplements and the liver tissues were assayed by the Carr-Price method and the vitamin A content of each evaluated from the standard reference curve. All determinations were made in duplicate and repeated if the deviation of duplicate values exceeded 3%.

RESULTS

Relation between the sex of the animal and liver storage of vitamin A

Using the method described in the preceding section depleted male and female animals were treated with 500 units per day of the vitamin A distillate. This experiment was repeated 4 times at different intervals. Using the per cent of vitamin A stored as the criterion, no difference was found between the males and females; males stored 32.5% whereas 31.5% of the ingested vitamin was found in the female livers (table 1).

In 1942 Brenner et al. reported studies concerning the relation of sex to vitamin A storage in the white rat. Their animals were fed 14,000 units of vitamin A daily for 35 days and then placed on a vitamin A-free diet. The liver stores of vitamin A were determined at the onset of the depletion period and at various intervals thereafter. The initial determina-

[1] Obtained from Distillation Products, Rochester, N. Y.

tion showed that the livers of females contained slightly greater amounts of vitamin A. After 1 week on the A-free diet the liver stores of males were only two-thirds those of the females when the results were computed as units of vitamin A per 100 gm of body weight. When the calculations were made on the basis of vitamin content of the total livers the difference was not as marked because males have larger livers. Brenner and associates concluded from these data that females have a greater capacity for storage of vitamin A.

TABLE 1

Relation between sex of animal and liver storage of vitamin A.

NO. RATS	SEX OF RATS	INTAKE OF VITAMIN A DISTILLATE NO. 78794	LIVER STORAGE OF VITAMIN A	STORAGE
		I.U./day	*I.U./liver*	%
6	Male	500	417	27.8
4	Male	500	434	28.9
6	Male	500	567	37.8
6	Male	500	530	35.3
			Average	32.5
6	Female	500	364	24.3
8	Female	500	500	33.3
6	Female	500	520	34.7
6	Female	500	507	33.8
			Average	31.5

The apparent discrepancy between Brenner's work and the foregoing experiment might be explained by the longer preliminary treatment period which he used in contrast with the 3-day treatment employed here.

Relation between intake of vitamin A distillate no. 78794 and storage in the liver of the rat

The following study was undertaken to determine the relative amounts of vitamin A stored in the liver as the intake was increased. Daily doses ranging from 63 to 118,400 I.U.

per rat were administered to depleted animals and the treatment continued for 3 days. The vitamin A distillate, used as a standard throughout this work, was the source of vitamin A.

The data in table 2 show that at the lowest dosage, 63 units per day, the per cent of the vitamin that was stored was relatively small (11.1%); but the values increased with larger doses until a maximum was reached at a level of 2,000 to

TABLE 2

Relation between intake of vitamin A distillate and storage in the liver of the rat.

NO. OF RATS	INTAKE OF VITAMIN A DISTILLATE NO. 78794	LIVER STORAGE OF VITAMIN A	STORAGE
	I.U./day	*I.U./liver*	%
5	63	21	11.1
6	125	92	24.5
6	250	215	28.6
6	500	480	32.0
18	1,000	1,032	34.4
6	2,000	2,220	37.0
6	4,000	4,530	37.8
5	8,000	8,000	33.3
6	16,000	13,900	29.0
5	32,000	21,200	22.1
5	48,000	25,400	17.6
6	64,000	29,950	15.6
6	80,000	31,800	13.3
6	118,400	49,200	13.9

4,000 units per day (37%). From this point on, although an increase in intake produced an increase in liver vitamin A, the percentage stored decreased until at 118,400 units per day only 13.9% of the dose administered was found in the liver. Thus, it is apparent that raising the daily intake above the optimum storage level did result in an increase in the amount of the vitamin in the liver but this was accompanied by a decrease in the percentage of the original dose that was stored.

Relation between the intake of vitamin A from various sources and storage in the liver of the rat

Samples of vitamin A from various sources were chosen in order to compare their relative storage efficiency in the liver. The oils tested included vitamin A distillate no. 78794 which has been discussed in a previous section; vitamin A acetate; two types of fish liver oils, halibut and cod; and a saponified vitamin A concentrate. The concentrate was a fish liver oil which had been saponified in order to separate the vitamin A from impurities present in the crude oil. The unsaponifiable fraction which contained vitamin A in the alcohol form was extracted with ether and then concentrated by evaporating the solvent.

Vitamin A from each of these sources was fed at 3 levels; 250, 500 and 1,000 units per day, and the percentage stored in the liver was determined. The summarized results in table 3 indicate that with the exception of some samples of cod liver oil, vitamin A was stored equally well regardless of the source. Cod liver oil no. 92995 produced slightly lower liver stores of vitamin A than did the other oils administered. It has been reported (Oser et al., '43; '45) that cod liver oils are relatively unstable and tend to oxidize, which might possibly explain the lowered liver storage values reported here. Oser et al. had noted changes in the shape of the ultra-violet absorption curve during the oxidation of vitamin A and found that an upswing of the left leg of the curve with a lower extinction coefficient at the absorption maximum of 328 mμ was typical of oxidized vitamin A. In order to check cod liver oil no. 92995 for evidence of similar deterioration an ultra-violet absorption curve was obtained but the shape was characteristic of vitamin A with a peak at 327 mμ, which seemed to indicate that no oxidative change had occurred. Four additional samples of cod liver oil were then selected and used for feeding experiments. Two of these (no. 93445, no. 8955) produced somewhat lower liver stores while the vita-

min A from cod liver oils no. 7065 and no. 8945 was stored as well as that from other sources investigated. All cod liver oils were assayed by colorimetric, biological and spectrophotometric methods to insure an accurate estimate of the potency. Good agreement was noted between the values obtained by the 3 methods indicating that accurate measurements of the vitamin A administered had been made.

TABLE 3

Relation between intake of vitamin A from various sources and storage in the liver of the rat.

NO. OF RATS	VITAMIN A INTAKE	VITAMIN A IN LIVER	STORAGE	NO. OF RATS	VITAMIN A INTAKE	VITAMIN A IN LIVER	STORAGE
	I.U./day	*I.U./liver*	%		*I.U./day*	*I.U./liver*	%
	Vit. A distillate no. 78794				Cod liver oil no. 93445		
6	250	215	28.6	5	250	83	11.1
6	500	480	32.0	6	500	358	23.9
18	1000	1032	34.4	6	1000	755	25.2
	Halibut liver oil no. 92295				Cod liver oil no. 7065		
6	250	238	31.7	6	250	214	28.5
6	500	487	32.5	3	500	550	36.7
6	1000	1060	35.3	5	1000	835	27.8
	Vit. A concentrate no. 5822				Cod liver oil no. 8945		
6	250	233	31.0	6	250	195	26.0
6	500	437	29.1	6	500	454	30.3
5	1000	962	32.1	6	1000	1008	33.6
	Vit. A acetate no. 94265				Cod liver oil no. 8955		
5	250	185	24.7	6	250	138	18.4
6	500	480	32.0	5	500	348	23.2
5	1000	890	29.7	6	1000	630	21.0
	Cod liver oil no. 92995						
6	250	155	20.7				
11	500	269	17.9				
12	1000	625	20.8				

It was, therefore, concluded that with the exception of some samples of cod liver oil which gave sub-optimal liver storage, vitamin A was equally utilized and stored whether fed as a distillate, acetate, cod liver oil, halibut liver oil, or as a saponified concentrate.

In 1940 Gray and associates published the results of somewhat similar experiments in which equal amounts of vitamin A as U.S.P. Reference Oil, vitamin A caproate, distilled ester concentrate, vitamin A stearate, vitamin A alcohol and β-carotene were administered. Their data showed that slightly lower liver storage resulted from treatment with the alcohol form of the vitamin but when the experimental error was taken into consideration the significance of this difference seemed questionable. Animals receiving β-carotene were found to have very low vitamin A reserves but the remaining preparations produced equal liver stores.

Storage of vitamin A in rat liver following oral, subcutaneous, or intramuscular administration

Vitamin A distillate no. 78794 at a level of 500 units per day was administered to depleted rats orally or injected, either subcutaneously or intra-muscularly, as shown in table 4. The oil preparation was diluted in corn oil so that the animals received 0.1 ml per day for the 3-day period. The same dilution was used for all modes of treatment. As in the previous experiments, no treatment was given on the fourth day and

TABLE 4

Storage of vitamin A in the liver of the rat following oral, subcutaneous, and intramuscular administration of vitamin A distillate no. 78794.

NO. RATS	LEVEL OF VITAMIN A GIVEN	MODE OF ADMINISTRATION	LIVER STORAGE OF VITAMIN A		STORAGE
	I.U./day		*I.U./liver*		%
6	500	Oral	543		36.0
5	500	Oral	525		35.0
				Average	35.5
6	500	Subcutaneous	150		10.0
4	500	Subcutaneous	217		14.5
				Average	12.3
4	500	Intramuscular	11		0.7
5	500	Intramuscular	6		0.4
				Average	0.6

the animals were killed by decapitation on the fifth day preliminary to the determination of vitamin A in the livers. Liver stores of vitamin A were believed to indicate the amount of the vitamin which had been absorbed by rats which were treated for 3 days and killed at the beginning of the fifth day.

Oral administration of the vitamin produced optimum liver storage; subcutaneous treatment allowed less storage and the intramuscular method was the least effective. Vitamin A administered subcutaneously was approximately 35% as effective as the same amount given orally; intramuscular injection was about 2% as efficient as oral treatment. A relatively short time was allowed for the absorption of injected vitamin A when the 3-day treating technique was used and it might be assumed that under these conditions the vitamin A would not be completely utilized. It can be seen, however, from the growth studies outlined below that daily injections of vitamin A continued for 4 weeks did not afford maximum utilization. An inferior response was observed when parenteral administration of vitamin A was employed irrespective of the time allowed for absorption of the injected oil.

Growth response to oral, subcutaneous, and intramuscular administration of vitamin A

Vitamin A-depleted rats were treated daily for 4 weeks with vitamin A distillate no. 78794 given orally, subcutaneously, or intramuscularly at 2 different levels (table 5) and the utilization of vitamin A was indicated by the increase in growth. The growth response data paralleled the liver storage data in that they showed oral administration to be superior to parenteral treatment.

In agreement with the present work it has been reported that vitamin A oils are more effective orally than when injected, both in supporting normal growth and promoting liver storage (Koehne and Mendel, '29; Greaves and Schmitt, '37; With, '39; Barlow and Kocher, '42; Lease et al., '42). Although most investigators have used lower levels of vitamin A

and continued the treatment for various periods ranging from 4 to 6 weeks, their results are in accord with those reported here.

The close correlation between weight response and liver storage in depleted rats following administration of vitamin A indicates that the measurement of liver reserves following a 3-day treatment period is a reliable method for judging the effectiveness of a treatment.

TABLE 5

Growth response following oral, subcutaneous and intramuscular administration of vitamin A distillate no. 78794.

GROUP	LEVEL VITAMIN A GIVEN	METHOD OF ADMINISTRATION	NUMBER OF RATS		AVERAGE WEIGHT GAIN (28 DAYS)
			Started	Finished	
	I.U./day				*gm.*
1	2.04	Oral	20	19	30
2	4.08	Oral	20	17	60
3	2.04	Subcutaneous	20	2	—9
4	4.08	Subcutaneous	20	4	3
5	2.04	Intramuscular	20	1	—21
6	4.08	Intramuscular	19	4	—7

SUMMARY

1. Equal liver stores of the vitamin were found in both male and female animals when depleted rats were treated for 3 days with large amounts of vitamin A.

2. The relative amount of vitamin A stored in the liver was determined after treatment with increasing doses. At the lowest intake of 63 units per day the liver storage was 11% of the total dose while maximum storage (37%) was obtained at a daily level of 2,000 to 4,000 I.U. Further increase in the amount of vitamin A administered was accompanied by a steady decrease in the efficiency of storage.

3. A comparison was made of the liver storage resulting from treatment with different types of vitamin A preparations. It was found that different lots of cod liver oil, although administered at equal unit levels, produced variable liver reserves of vitamin A. With the exception of some lots of cod

liver oil which gave sub-optimal storage, vitamin A was utilized and stored equally well whether fed as a distillate, acetate, halibut liver oil, or saponified concentrate.

4. The storage of vitamin A in the livers of rats following oral, subcutaneous, or intramuscular administration was investigated. The oral method was found to be most effective in producing liver storage. Vitamin A injected subcutaneously was approximately 35% as efficient over a 5-day period as the same amount given orally, while intramuscular injection was only 2% as effective as the oral route.

5. Depleted rats were treated daily for a period of 4 weeks with vitamin A given orally, subcutaneously, and intramuscularly and the increase in growth was measured. It was found that vitamin A distillate was more effective orally than parenterally in promoting growth as well as in producing liver stores.

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STUDIES IN AMINO ACID UTILIZATION

I. THE DIETARY UTILIZATION OF MIXTURES OF PURIFIED AMINO ACIDS IN PROTEIN-DEPLETED ADULT ALBINO RATS [1]

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FOUR FIGURES

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The substitution of mixtures of purified amino acids for dietary protein in rations adequate in calories, vitamins and minerals has helped greatly to clarify understanding of the nutritive potentialities of proteins. It is now possible to prepare a mixture of the 10 so-called essential amino acids which promotes growth in young rats (Kinsey and Grant, '44), or mice (Bauer and Berg, '43), or maintains nitrogen balance in dogs (Rose and Rice, '39) and adult man (Rose, Haines, Johnson and Warner, '43). Many questions remain unanswered, however, concerning the mechanisms governing the utilization of these amino acids. The question of the indispensability of arginine and histidine needs further study.

[1] The subject matter of this paper has been undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the armed forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

The work has been aided, also, by the John and Mary R. Markle Foundation, The National Livestock and Meat Board, The Douglas Smith Foundation for Medical Research of the University of Chicago, and the Allen B. Wrisley Company.

More information is needed regarding such problems as the possible toxicity of some of the unnatural forms (Albanese and Irby, '43), the optimal amounts of each amino acid in the daily ration, the role of accessory substances such as strepogenin (Woolley, '45) in the synthesis of proteins from amino acid mixtures, and the possible adverse effects from the use of improperly-balanced mixtures even though all of the indispensable amino acids may be present.

In this and the following paper all observations were made on a group of protein-depleted rats fed rations whose nitrogen source, other than traces in the cornstarch and vitamins, consisted of a mixture of either 16 (ration A), 10 (ration B), or 9 (ration C), purified amino acids. In these 2 papers we present evidence concerning the influence of these rations upon (a) weight-recovery; (b) food consumption; (c) plasma and red cell volumes and regeneration of serum proteins and erythrocytes. Although the data are from experiments dealing with only 28 rats, they are presented as illustrative examples. As a matter of fact, the experiments have been confirmed with 55 additional rats so that, in reality, they represent findings from the use of more than 85 different animals.

The "sixteen amino acid mixture" utilized was patterned after the amino acid composition of casein (Block and Bolling, '45), except that serine, proline, and hydroxyproline were absent and the racemic mixtures of threonine, valine and isoleucine were used with the assumption that the unnatural *d* forms do not replace their respective enantiomorphs metabolically. Although the carbon chains of these *d* amino acids may not be used for the synthesis of tissue protein, their amino nitrogen may be utilizable for the synthesis of nonessential amino acids. Accordingly, an amount of glutamic acid equivalent to the non-utilizable *d* forms was omitted from the mixture. Correction was made for HCl in the arginine, histidine and lysine samples and for water of crystallization in the latter two. Whenever an amino acid was omitted from the mixture in order to observe the effect of its deficiency, its

equivalent in moles of glutamic acid was substituted. When amino acid mixtures were made containing only 10 or 9 essential amino acids, the ratios of the amino acids were held constant while their concentrations were increased in order to keep the dietary nitrogen levels the same. The amounts of

TABLE 1

Composition of amino acid mixtures employed to supplement basal ration 4 E.

	PERCENTAGE COMPOSITION OF MIXTURES OF AMINO ACIDS IN			MG OF ESSENTIAL (1) AMINO ACIDS PER 15 GM DIET PER RAT DAY IN		
	Ration A	Ration B	Ration C	Ration A	Ration B	Ration C
l (+) Arginine HCl	4.214	6.592	.....	63.4	99.1	....
l (+) Histidine HCl	2.871	4.491	4.808	38.5	60.4	64.7
dl Isoleucine	11.050	17.286	18.505	100.0	157.2	168.3
l (+) Leucine	10.285	16.186	17.224	190.0	294.3	313.1
l (+) Lysine HCl	8.051	12.594	13.483	106.0	167.2	178.5
dl Methionine	2.975	4.654	4.982	54.0	84.6	90.6
dl Phenylalanine	4.420	6.914	7.402	80.0	125.7	134.6
dl Threonine	6.630	10.371	11.103	60.0	94.3	100.9
dl Tryptophane	1.530	2.393	2.562	27.7	43.5	46.6
dl Valine	11.900	18.615	19.929	108.0	169.2	181.2
dl Alanine	4.760					
dl Aspartic acid	5.355					
l (—) Cystine	0.306					
l (+) Glutamic acid	20.157					
Glycine	0.425					
l (—) Tyrosine	5.440					
	100.069	99.999	99.998			

each amino acid present in the different mixtures are given in table 1.[2] In the graphs, the ration containing 16 amino acids was originally referred to as AAR. For greater convenience it is referred to in these papers as ration A. Similarly, the ration containing the 10 "essential" amino acids, referred to

[2] Most of the amino acids were purchased from Merck and Co. Alanine, glycine, isoleucine, threonine, tyrosine and valine were purchased from General Biochemicals, Inc. Some methionine was purchased from the Pfanstiehl Co. Purity of the amino acids was checked by Kjeldahl determinations in the case of the racemic forms and by optical rotation determinations in the case of the active natural forms.

in the graphs as EAAR10 or EAAR, is here called ration B; and the 1 containing only 9 essential amino acids, shown in the graphs as 9AA or 9EAAR, is here called ration C.

EXPERIMENTAL PROCEDURES

Male, albino rats of the Sprague-Dawley strain, with initial weights varying from 220 to 244 gm, were subjected to protein depletion according to procedures previously described (Wissler, Woolridge, Steffee and Cannon, '46). Selection was made from groups of rats which had been on a low protein "depletion" diet (3E) for approximately 3 months, animals being chosen on the basis of the uniformity in initial weights, percentages of weight loss (25–33%) and concentrations of serum proteins (4.06–4.85%) and hemoglobin (11.2–15.3%). The rats were placed in individual cages prior to the incorporation of the amino acid mixtures or casein into the basal ration for use in the "repletion" period. All rations were kept isocaloric. Two days before the experiments were to be started the animals were weighed, and bled (tail vein) in order to determine the serum protein and hemoglobin concentrations. On the basis of these data as well as the initial predepletion weights and percentage weight losses, the most comparable animals were paired, 1 animal of each pair being offered a weighed amount (15 gm per rat per day) of ration A, while its mate was fed this ration with one "essential" amino acid omitted. Water was offered ad libitum. For comparison, other pairs of rats were fed the 4E basal or casein rations under similar circumstances and concentrations of nitrogen (table 2). Blood volumes were determined on the first day of the experiment. During the first dietary period the amounts of ration eaten, wasted or rejected were determined daily and the animals were weighed frequently. On the tenth day the rats were fed in the morning but were fasted for the last 12 hours of the 24-hour period. On the morning of the eleventh day, weights, serum protein and hemoglobin concentrations and blood volumes were determined. In most instances thereafter the diets of each pair of animals were reversed for a

second 10-day period during which weights and diet-consumption figures were determined as before. At the end of the 20-day period the animals were again fasted for 12 hours and the various observations repeated after which they were sacrificed and autopsied.

TABLE 2

Composition of rations used.

INGREDIENTS PER 100 GM	4 E BASAL	CASEIN	AMINO ACIDS
Nitrogen source	0.0	10.46	12.12
Corn oil	4.0	4.0	4.0
Cornstarch	71.0	61.7	58.88
Ruffex	5.0	5.0	5.0
Salt mixture [1]	4.0	4.0	4.0
Vitamin solutions	2.7	2.7	2.7
Water	13.3	12.14	13.33
VITAMINS PER 100 GM OF RATION			
Choline chloride	0.20 gm		
Oleum percomorphum	0.30 drops		
Nicotinamide	2.84 mg		
Pantothenate Ca	1.75 mg		
Pyridoxine HCl	0.64 mg		
Riboflavin	1.08 mg		
Thiamine HCl	0.54 mg		

[1] Hawk and Oser's ('46) modification of the Osborne and Mendel salt mixture plus 1 gm each of copper sulfate, and zinc chloride added to the trace elements.

The utilizable amounts of each of the essential amino acids supplied per 15 gm of ration for the "amino acid rations" are given in table 1. From these data it is possible to compute the milligrams of amino acids eaten per rat day, knowing the actual amount of food consumed.

EXPERIMENTAL FINDINGS

The effects of ration A upon weight-recovery are shown graphically in figure 1, where the starting weights have been connected with the 10-day and 20-day values by straight lines. It will be noted in every instance that the animal which ate this ration for 10 days gained weight rapidly, an average of

between 4 and 5 gm per day, whereas each animal which ate the ration with any one of 9 indispensable amino acids omitted continued to lose weight. Furthermore, comparison of these results with those of a sample pair of the animals which ate the 4E basal ration alone or this ration supplemented with casein (fig. 2) shows a less rapid weight recovery for the rat which ate the natural protein than for several of those consuming ration A. Moreover, the animals which ate the 4E ration alone lost less weight, in general, than did the animals which ate the ration containing fifteen amino acids but with 1 indispensable amino acid omitted. In other words, the animals did slightly better on no protein at all than on a mixture of 15 amino acids but with 1 indispensable amino acid lacking. Exception occurred only with respect to lysine deficiency, where weight loss was practically identical with that for the rats fed the 4E ration. With respect to arginine, its presence or absence seemed to make but little difference. One rat, in fact, kept on an arginine deficient diet for 30 days, recovered almost as much lost weight as did its companion which ate ration A; furthermore, spermatogenesis as judged by histological study was as well reestablished in the arginine-deficient rat as in the other. It is obvious, therefore, that the protein-deficient rats were able to synthesize arginine with sufficient rapidity almost to keep pace with those supplied with arginine.

In view of the unsuccessful use by Albanese and Irby ('43) of a mixture of 10 amino acids for growth of young rats, and because of their suggestion of the possibility of toxic *d* forms being present in such a mixture, we prepared a mixture of 10 amino acids (table 1) and incorporated it in the basal ration at the same nitrogen concentration employed for ration A. Figure 2 demonstrates the results obtained from the feeding of these 2 types of rations. It is obvious that weight recovery was as good with only 10 amino acids in the ration as with the 16 of ration A. It is evident, also, that there was no apparent "toxic" effect from the use of a mixture containing several of the unnatural *d* forms.

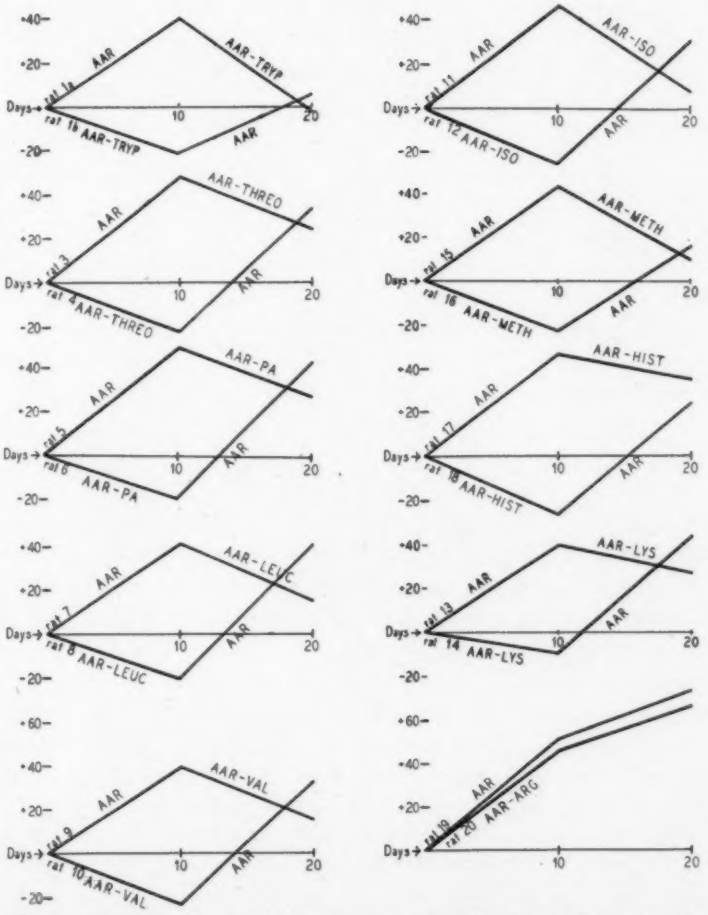

Fig. 1 Comparison of weight changes in protein-depleted rats fed ration A (AAR) and ration A devoid of 1 indispensable amino acid. After 10 days the rations were reversed. Note the common pattern except in the case of arginine.

Although it is apparent that a protein-deficient rat can recover lost weight when only 10 amino acids are present in the ration, we wished to secure additional evidence regarding the dispensability of arginine. Accordingly, a mixture of amino acids containing only 9 indispensable amino acids was prepared (table 1, ration C), but, again, with as much total nitrogen as was present in rations B and A. This mixture when added to the basal ration, at a comparable concentration of nitrogen, yielded essentially as good a weight recovery rate as did ration A, fed simultaneously (fig. 2).

One of the most striking features of the experiments in which the rats were offered rations deficient in any one of the 9 indispensable amino acids, was their disinclination to consume the ration. In other words, these 9 amino acids are essential, also, for good appetite, and omission from the ration of any 1 of them, even though its nitrogen equivalent is replaced by glutamic acid, is followed quickly by reduced food-consumption. On the other hand, addition to the ration of the missing amino acid, is followed just as promptly by improved appetite. The data, which record the amounts of food consumed, are shown graphically in figures 3 and 4 in which the rectangles are designated "food-consumption areas." The 4 rectangles for each amino acid tested represent the proportions of ration (15 gm per rat per day) eaten daily by the protein-depleted rats for each 10-day period. The upper left-hand rectangle represents the amount of food eaten by the rat fed ration A, the lower left hand one, the amount of food eaten after 1 amino acid had been omitted from the ration.

Fig. 2 (Upper left). Comparison of weight changes in 2 rats, one of which was fed ration A (AAR) and the other ration B (EAAR10), both for 20 days. (Upper right). Weight changes in 2 rats one of which was fed ration A for 20 days (no. 211–4) and the other (no. 214–1) one fed this ration with the lysine injected subcutaneously daily for 10 days and then replaced by salt solution. (Lower left). Weight changes in rats fed casein ration and protein-free ration for 10 days and after reversal of rations. (Lower right). Comparison of weight changes in 2 rats fed ration A and ration C (9AA) for 20 days. All rats were protein-depleted at the beginning of the experiments.

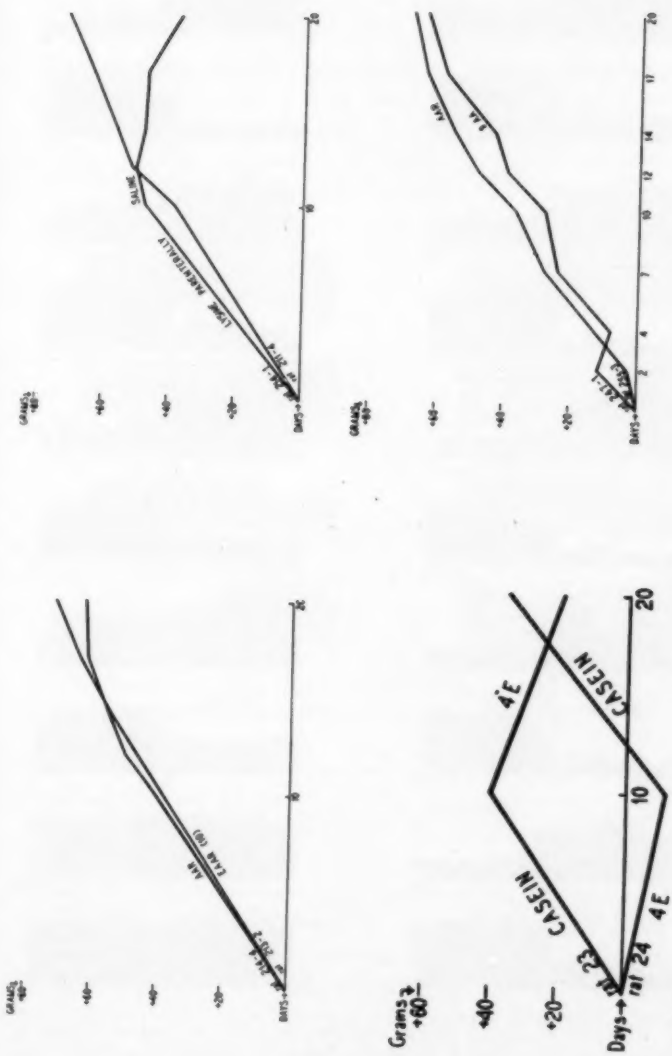

Figure 2

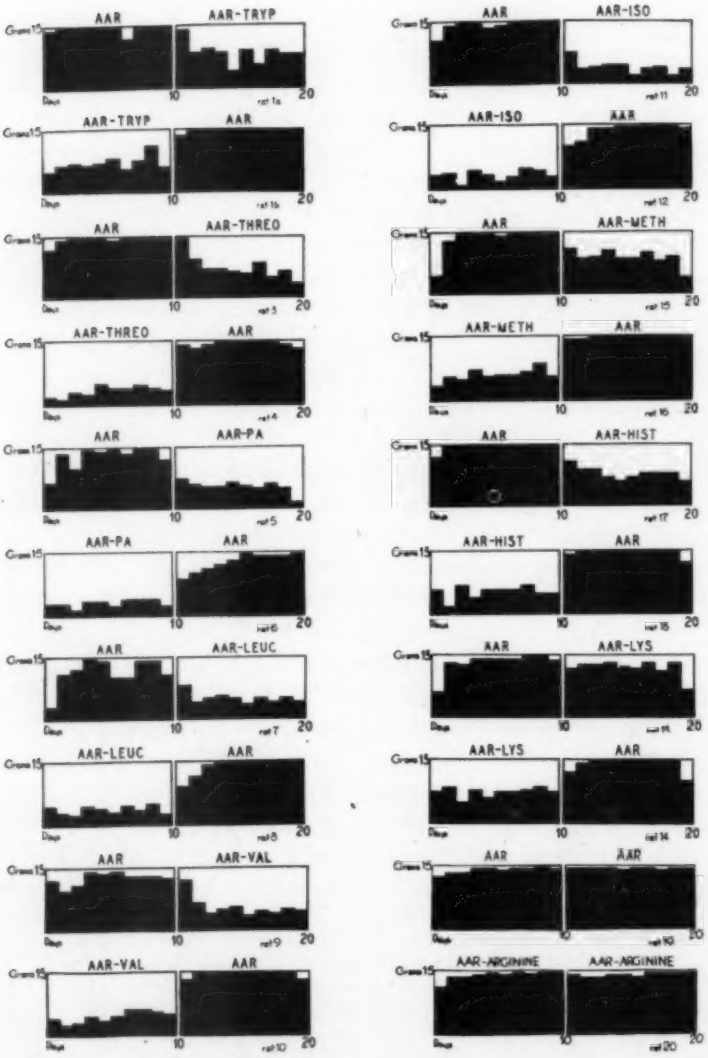

Fig. 3 "Food consumption areas" resulting from the feeding of rations, to be examined in relation to figure 1. Note the common pattern except in the case of arginine.

The 2 rectangles on the right indicate amounts of food consumed by each rat for a second 10-day period after reversal of the rations. Inspection of the rectangles demonstrates in every instance that food-acceptance is correlated with the presence or absence of 1 of the 9 indispensable amino acids.

A more detailed examination of these "food consumption areas" will illustrate more definitely the effects observed. For example, rats 1a and 2b were offered rations identical except

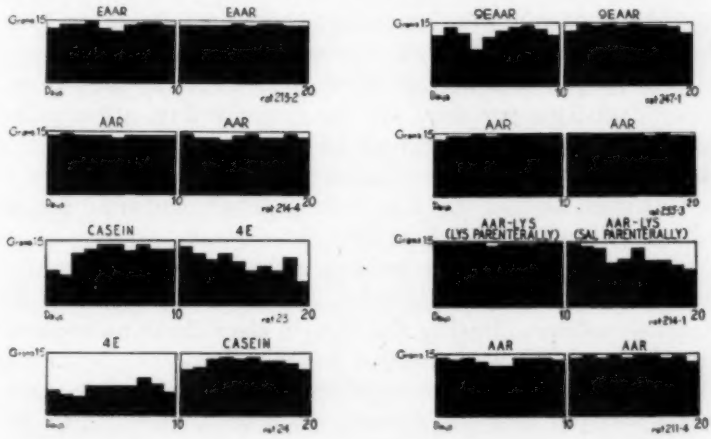

Fig. 4 "Food consumption areas" to be compared with weight changes of figure 2 in rats fed the various types of rations.

for the presence or absence of approximately 29 mg of dl-tryptophane per 15 gm of ration. As can be seen, rat 1a ate practically all of his daily ration for the first 10 days. During this time he recovered lost weight. On the eleventh day, although tryptophane was now absent from the ration, he ate 14 gm; but on the twelfth day he ate only 8.5 gm, and his daily food-consumption thereafter was always less than 10 gm. During this time he lost weight steadily. In contrast, rat 1b which was offered the ration devoid of tryptophane, ate poorly for the entire 10-day period and on only 1 day (the ninth) did he eat more than 10 gm. On the eleventh day, after tryptophane

had been added to the ration, he ate 13.5 gm, and on each of the succeeding 9 days he ate all of the ration; during this period he gained weight steadily. Rats 9 and 10 reacted similarly when valine was tested, After 10 days' consumption of ration A, rat 9 had made a good weight-recovery and had eaten the ration well. On the eleventh day, after approximately 216 mg of dl-valine had been removed from the ration, the rat ate the same amount of food which he had eaten on the preceding 4 days (12 gm per day). On the twelfth day, however, his food-consumption dropped to 6.5 gm and stayed low for the succeeding 8 days. In contrast, rat 10, when first offered the ration devoid of valine, refused for 10 days to eat more than 6 gm per day. On the eleventh day, after valine had been added to the ration, he ate 13 gm, and from then on he ate his daily ration almost completely. A similar pattern is seen, also, in the case of the 2 rats (numbers 3 and 4) offered rations with and without threonine.

Of interest, also, are the findings in the rats which responded to the addition of phenylalanine, leucine or isoleucine to the deficient rations. During the first 10 days these animals refused to eat well when offered rations devoid of any one of these amino acids. When, however, each of the missing amino acids was put into the rations, daily food-consumption improved in a step-wise fashion until practically maximal consumption was attained and maintained. These patterns suggest a gradual return of appetite, associated with a gradually increased ingestion of those amino acids in which the rations had been deficient. It is noteworthy, also, that this response occurred when such dl- forms as phenylalanine, valine, isoleucine and threonine were used, thus indicating the lack of any toxic effect from the unnatural forms supplied, as judged by appetite or weight recovery.

In an effort to ascertain whether these changes in appetite might depend upon taste, 2 animals were given rations differing only in the route by which lysine was administered, viz., 1 rat was fed ration A for 20 days while the second rat was fed this ration devoid of lysine but with the lysine injected sub-

cutaneously, 2 doses per day (146 mg 1 (+) lysine $HCl \cdot H_2O$ per rat day). As can be seen from figure 2, the rat which received lysine parenterally made an even better weight recovery than did the one which ingested lysine. At the end of 10 days, however, when salt solution was substituted for the injected lysine, the animal began quickly to lose weight, whereas the rat eating ration A continued steadily to gain weight. Evidence of this kind speaks against the idea that taste may be a determinant of appetite under these circumstances; on the other hand it does not eliminate the possibility that food consumption may be influenced by blood amino acid thresholds, at least until such a possibility can be studied more completely.

The degree of consumption of rations other than ration A is shown in figure 4. Examination of these "food-consumption areas" indicates that the protein-depleted rats maintained good appetites for 20 days when fed rations containing only 10 or 9 indispensable amino acids as the principal source of dietary nitrogen. Their food intake, in fact, equalled that of the animals eating the casein ration and exceeded that of the rats eating the basal ration. It is obvious, also, that when lysine was injected, the rat's appetite was as good as that of the animal eating the complete ration A but that appetite declined when salt solution was substituted for the injected lysine.

COMMENT

Although it has long been known that a loss of appetite may at times be an indicator of an amino acid deficiency, we have found no statements emphasizing the rapidity with which this may manifest itself. Rose ('38) has said that "growing animals lose the desire to eat when the food is not suitable for tissue-synthesis, but regain it when all of the components required for anabolism are made available" and that (Rose, '31–'32), "Our experience with other types of deficiencies involving the nitrogenous portion of the ration has taught us to expect a marked failure in appetite when the diet is completely devoid of an essential component . . . Hundreds of

experiments . . . involving the use of completely hydrolyzed proteins, have demonstrated that rats readily consume adequate amounts of diets in which the nitrogenous portion is in the form of mixtures of amino acids, provided all of the materials for growth are available." Smith ('31) has also remarked that "an experimental animal deprived of protein soon exhibits a complete loss of appetite."

Our findings corroborate these statements. They also demonstrate the surprising fact that food-acceptance by protein-depleted and presumably hungry rats may be determined by the presence or absence of only a few mg of a particular indispensable amino acid. For example, in experiments to be reported in more detail later, we found that a rat offered a ration containing approximately 18 mg of dl-tryptophane per 15 gm of ration ate almost all of the daily ration and gained 43 gm in 10 days. Another rat, however, offered the same ration containing only 9 mg of dl-tryptophane per 15 gm of ration had an average daily food-consumption for the 10-day period of 10 gm and gained but 4 gm of weight. In other words, the difference in weight recovery of the 2 animals was determined by the presence or absence in the daily ration of only 9 mg of dl-tryptophane. Similar effects have been observed with the other 8 indispensable amino acids although the quantitative requirements for each amino acid are different.

The mechanism governing this impairment of appetite in acute amino acid deficiency is of especial interest. Is it the result of a lowered taste-threshold due to the lack of certain amino acids, or is it due to a general metabolic disturbance whereby the sense of well-being of the animal is altered, as a result of which the rat becomes actually sick and hence is not interested in food? If the loss of appetite is due to inadequate stimulation of taste organs, it would appear necessary to assume the existence of 9 indispensable amino acid "essential," also, for taste and that omission of any one of them from the diet would depress the taste stimulus for all the others. Against the idea of taste, however, is the fact that in

several of our experiments the animal continued to eat well for 1 day after removal of the indispensable amino acid from the ration, after which food-consumption declined and remained low. If taste were the determining factor here it is difficult to see why it should require 24 hours for the rat to discover the absence of a particular amino acid from the ration and then to eat only one-half or less of his daily diet.

Evidence suggesting a general metabolic disturbance as the factor responsible for the impaired appetite is furnished by experiments (Rose et al., '42; '43) in which healthy young men were fed rations adequate in calories, vitamins and minerals but containing a mixture of 10 essential amino acids as the only source of dietary nitrogen. The men remained in nitrogen balance until any 1 of 8 amino acids was removed from the ration whereupon they went promptly into negative nitrogen balance. Readdition to the ration of the missing amino acid, however, brought them back quickly into nitrogen balance. Of especial interest is the fact that when the subjects were in negative nitrogen balance they felt miserable and had impaired appetites but that after nitrogen balance was reestablished they again felt better and their appetites improved.[3] It is possible, therefore, that a similar mechanism may operate in the rats and that their disinclination to eat may be due largely to the fact that they actually feel too "sick" to be interested in food, even though they are severely undernourished and need the food to promote effective convalescence.

The possibility should also be considered that the loss of weight and the associated impairment of appetite accompanying acute amino acid deficiency might be due, indirectly, to the restricted intake of calories, vitamins and minerals. In order to test this possibility, protein-depleted rats were gastrostomized and then fed through the gastrostomy tube. Although these experiments are still in progress, evidence obtained thus far indicates that an adequate intake of calories,

[3] Personal communication from W. C. Rose.

vitamins and minerals lessens but does not prevent the weight-loss of acute amino acid deficiency. For example, 1 rat fed the ration A via gastrostomy for 10 days gained weight steadily; but when tryptophane was removed from the ration he began to lose weight despite the fact that his caloric, vitamin and mineral intakes were unchanged. However, his weight loss was less than that of his control being offered the deficient ration ad libitum, thus suggesting that if acute amino acid deficiency induces some sort of "toxic" disturbance of internal metabolism, this is less severe in the presence of adequate calories, vitamins and minerals. At any rate, the mechanism responsible for the impaired appetite requires further study.

These consequences of acute amino acid deficiency emphasize further some of the difficulties which hamper efforts to evaluate quantitatively by biological methods the nutritive potentialities of proteins. If only a few mg of any 1 of 9 indispensable amino acids can determine either nutritive success or failure of a biological assay, it is not surprising that there should be so much controversy concerning the kinds of methods which should be utilized for determining protein quality. There is still no general agreement as to the nitrogen level optimal for protein-assay; and no biological method has as yet been developed which can determine the concentrations of each individual amino acid in a test-ration. Ultimately, it may become necessary to ascertain the limiting level of each indispensable amino acid in the protein being tested rather than to determine this indirectly as is now done. Further improvements in methods of amino acid analysis, both chemical and microbiological, may make this possible. Thus it may be possible to demonstrate absolute quantities of indispensable amino acids in a protein and their ratios one to another. Similarly, it may be possible to relate these values to those for the nonessential amino acids as well. However, even then, biological methods will still be needed because of the fact that, as a consequence of heat-injury or processing alteration, certain indispensable amino acids may be rendered metabolically

non-utilizable even though they are demonstrable by amino acid analysis.

It is of interest that these experiments failed to demonstrate the retarded weight gains observed by others (Woolley, '45; Rose and Womack, '46) in young animals fed rations whose nitrogenous constituents came from hydrolyzed protein or from mixtures of purified amino acids. Although we know of no source of strepogenin in the materials used in the rations of our experiments it should be mentioned that the ration 3E, used for the depletion period, contained 1% of Wilson's 20:1 liver concentrate. It is possible, therefore, that strepogenin from this source might have been stored in the depleted rats so that no deficiency was apparent in the relatively short "repletion" period. Cary et al. ('46) have presented evidence that an unidentified accessory growth factor present in liver, casein and some other foods, may be stored by young rats. Our rats are almost full-grown and may not need exogenous accessory factors as do young rats. At any rate, our experiments agree with those of Rose that, with the exception of arginine, the same 9 amino acids essential for adequate growth in the young rat are also essential for effective weight-recovery and good appetite in adult protein-depleted rats.

SUMMARY

Weight-recovery of comparable pairs of protein-depleted rats was determined while the animals were fed "amino acid rations" for a 10-day period. In most instances 1 rat was offered a ration containing a mixture of 16 amino acids patterned after the amino acid composition of casein while the second received the same mixture with one indispensable amino acid omitted. In most cases the rations were reversed for a second 10-day period. In some experiments fewer amino acids were employed. The results may be listed as follows:

1. Rats fed the amino acid ration containing 16 amino acids (ration A) gained weight rapidly, usually exhibiting as great or greater weight gains in 10 days as rats fed a ration con-

taining an isonitrogenous quantity of casein; they also ate the ration completely.

2. Omission, singly, of histidine, lysine, tryptophane, phenylalanine, methionine, threonine, leucine, isoleucine or valine from this ration led to marked loss of weight in a 10-day period, coincident with a prompt loss of appetite as evidenced by consumption of only from one-third to one-half of the daily ration offered. The weight loss in most cases was greater than that shown by comparable rats fed a low-protein basal ration for a similar period. When, however, the missing amino acid was added to the ration the rats quickly recovered lost appetite and rapidly regained lost weight.

3. When arginine was omitted from the ration the rats gained weight at rates approximately equal to those of similar rats receiving the complete amino acid ration and their appetites were well-maintained. No histological differences in spermatogenesis could be seen after 30 days' feeding of the respective rations.

4. A ration containing only the 10 amino acids indispensable for rat growth, fed at a nitrogen level equivalent to that in ration A, supported a weight gain equal to or greater than that of similar rats receiving the 16 amino acids. Similarly, when only the 9 amino acids listed above were fed, an equivalent rate of weight gain was obtained. With both types of ration good appetites were maintained over a 20-day period.

5. When 1 amino acid (lysine) was administered parenterally and the others were consumed by mouth, the weight increase was comparable to that of rats receiving the complete ration by mouth; good appetite was also maintained. When, however, salt solution was substituted for the lysine, both the animal's weight and appetite declined.

6. The rapidity with which appetite declines in these protein-depleted rats after omission of any one of 9 indispensable amino acids from the ration suggests the development of a state of acute amino acid deficiency which can be corrected only by the addition to the ration of adequate amounts of the missing amino acid. Furthermore, no evidence of a need for

an accessory polypeptide factor or factors to promote effective utilization of these amino acids was demonstrated by these experiments in which the only supplementary constituents were pure crystalline vitamins.

7. In the biological evaluation of protein, dilution of the test-protein to the point where acute amino acid deficiency occurs prevents an adequate quantitative estimation of nutritive quality. It is suggested that ultimately it may become necessary to evaluate proteins in terms of the limiting action of each indispensable amino acid in the test-protein rather than to attempt to evaluate amino acid levels indirectly by current methods.

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STUDIES IN AMINO ACID UTILIZATION

II. ESSENTIAL AMINO ACIDS AS A SOURCE OF PLASMA PROTEIN AND ERYTHROCYTES IN THE HYPOPROTEINEMIC RAT [1]

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Because of the great interest in plasma protein production in acute and chronic states of hypoproteinemia, and in the correction of these conditions by means of orally and parenterally administered mixtures of amino acids, it is important to know which amino acids are necessary for the production of plasma proteins. In the preceding paper the effects of various combinations of amino acids and of single amino acid deficiencies on weight recovery and diet consumption of the protein-depleted rat have been described (Frazier et al., '46). In the present paper the influence of various amino acids on the fabrication of serum protein and of erythrocytes is described.

EXPERIMENTAL PROCEDURES

The present observations were made on the same animals used in the experiments described in paper I of this series. At the end of the protein-depletion period the rats were paired

[1] The subject matter of this paper has been undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

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as described (Frazier et al., '46) and on the first day of the experimental period blood volume, serum protein and hematocrit determinations were made. The rats were then placed upon the test rations. At the end of 10 days these values were again determined, the diets of each pair of animals interchanged and a third set of values obtained after a second 10-day period.

Total circulating serum protein (T.C.S.P.) and total circulating erythrocyte volumes (T.C.E.V.) were computed from the determined values of total blood volume, plasma volume, serum protein and hematocrit. The determination of the blood volume using Evans Blue dye has been described in detail elsewhere (Benditt, Straube and Humphreys, '46). The method as used in the present experiments differs only slightly from that described. The points of difference are (a) in the dilution of the blood used for estimation of the dye concentration, and (b) the protein used in the diluting fluid. In the present investigations 0.2 ml of oxalated whole blood was diluted with 5.0 ml of diluting fluid which contained 2.0 gm of whole dehydrated beef plasma instead of the 2.0 gm of purified beef albumin described elsewhere. Serum proteins were determined by the method of Barbour and Hamilton ('26). Hematocrits were determined in capillary tubes as described.

From the measured concentration of dye in 0.2 ml of blood the total blood volume was calculated, and from this and the hematocrit value the total circulating red cells and the plasma volume were estimated. From the plasma volume and serum protein concentration the total circulating serum proteins were calculated.

RESULTS

In table 1 are presented the mean values and their standard errors for the quantities of plasma volume, serum protein concentrations, total circulating serum protein (T.C.S.P.), total circulating red blood cell volume (T. C. E. V.) and the body weight of a group of 8 animals fed the protein-free basal ration (4E) for 10 days, and another group of 7 animals fed the 9% casein ration (Cas) for 10 days. These data show a rea-

sonable constancy and compare favorably with other published figures on the same quantities measured in the rat (Metcoff and Favour, '44).

Table 2 presents the total circulating serum protein changes for the animals on the various diets. Column 1 contains the

TABLE 1

Averaged data for rats fed basal 4E and 9% casein rations.

	PLASMA VOLUME		SERUM PROTEIN		TOTAL CIRCULATING SERUM PROTEIN		TOTAL CIRCULATING RED CELL VOLUME		BODY WEIGHT	
	Day 0	Day 10	Day 0	Day 10	Day 0	Day 10	Day 0	Day 10	Day 0	Day 10
	ml		*gm %*		*mg*		*ml*		*gm*	
Protein free (4E) diet (8 animals)										
Mean	6.31	5.90	4.08	4.40	258	257	3.81	3.73	160	145
S.E.	± .72	± .51	± .59	± .37	± 14	± 13	± .52	± .66	± 2	± 2
9% casein (Cas) diet (7 animals)										
Mean	6.44	7.64	4.20	5.81	271	447	3.67	5.71	159	197
S.E.	± .66	± .71	± .66	± .44	± 15	± 19	± .66	± .65	± 2	± 2

TABLE 2

Changes in total circulating serum protein.

RATION	4E	CAS	RATION A	RATION A MINUS	RATION A MINUS	RATION A	
PERIOD (day)	0–10	0–10	0–10	10–20	0–10	10–20	
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*The missing amino acid*
	— 11	212	210	— 73	27	159	Tryptophane
	— 36	186	. . .	. . .	— 96	236	Lysine
	— 1	159	143	— 66	— 15	236	Methionine
	25	221	196	— 44	40	108	Histidine
	2	144	105	— 12	25	175	Threonine
	14	185	145	— 127	— 7	184	Valine
	4	111	151	— 60	45	178	Leucine
	0	. . .	229	— 110	11	175	Isoleucine
	. .	. . .	143	— 49	35	137	Phenylalanine
	.	. . .	137	. . .	151 [1]		Arginine
Ave.	0	174	162	— 68	7	176	
S.E.	± 20	± 15	± 13	± 13	± 15	± 15	

[1] The value for the arginine-deficient animal was excluded from the average.

values for the animals fed the 4E diet for the first 10 days, column 2 the values for the animals fed the Cas diet for the same period. In column 3 are the gains of the animals fed the complete amino acid mixture (ration A) for the first 10 days following depletion. Column 4 contains the data for these same animals during the second 10-day period (10–20) during which they were fed a diet lacking in one of the amino acids. The missing amino acid is indicated in the extreme right-hand column. The fifth column contains data on the animals paired with those represented in the preceding 2 columns and fed the deficient amino acid ration in the first 10 days; column 6 contains the data for the same animals when fed ration A in the second 10-day period.

Rats on the 4E diet for the first experimental period (0 to 10 days) had a mean change of 0 ± 20 mg in their T.C.S.P. Over a similar 10-day period the 7 animals on the Cas diet gained 174 ± 15 mg of protein in their circulation, almost doubling the amount which they had initially. With these values we can compare the performance of rats fed the other experimental diets.

All animals fed ration A during the first 10-day period gained considerable quantities of total circulating serum protein. The mean gain was 162 mg with a standard error of ± 13 mg. In the second 10-day period when these same animals were fed diets lacking one of the essential amino acids they all lost serum protein. The smallest loss was 12 mg, the largest 127 mg, the average 68. It is evident that after an initial feeding period the lack of one essential amino acid induces a greater loss in serum protein than in the animal at the end of a long period of protein depletion. The reason for this is not clear. It may be due to an increased metabolic rate related to the repletion of body proteins and enzyme systems during the preceding 10-day period.

When the initial diets lacked one of the essential amino acids, excluding arginine, diet consumption was markedly reduced and there were minor losses or gains of total circulating serum proteins of the order of those observed in the animals

on the 4E diet. There was one exception, the animal fed ration A minus lysine apparently lost 96 mg of protein. The mean change for the group of 9 animals was + 7 ± 15 mg. These same animals in the second 10-day period eating ration A gained on the average 176 ± 15 mg of protein. The smallest gain recorded was 108 mg by the rat fed the histidine deficient diet during its initial period.

The data (tables 2 and 3) on the animals used to test the indispensability of arginine show a striking difference from the data on the preceding 9 amino acids. The rat on the diet completely lacking arginine gained 151 mg of serum protein

TABLE 3

Total circulating serum protein and total circulating erythrocyte volume values for animals on diets lacking arginine and their controls.

RAT NO.	DIET	TOTAL CIRCULATING SERUM PROTEIN				TOTAL CIRCULATING RED CELL VOLUME			
		Day				Day			
		0	10	20	30	0	10	20	30
		mg	*mg*	*mg*	*mg*	*ml*	*ml*	*ml*	*ml*
154–2	Ration A	256	393	479	536	3.6	6.1	6.9	6.4
255–1	Ration A	257	456	519	. . .	3.6	5.7	6.3	. .
153–4	Minus arginine	282	433	488	559	3.3	4.8	6.8	6.2
247–7	Ration C	246	424	440	. . .	3.3	5.3	5.6	. .
247–3	Ration C	259	429	449	. . .	3.4	5.3	5.3	. .

in the first 10-day period. Its control on ration A gained 137 mg. In this instance the diets were not interchanged. In the second 10-day period the animal lacking arginine gained an additional 55 mg. After 30 days the test animal and its control had almost identical T.C.S.P. and T.C.E.V. values.

Data on the behavior of the total erythrocyte mass are presented in table 4. With no protein in the diet there was an average loss in 10 days of 0.1 ± 0.2 ml in total circulating erythrocytes. With 9% casein there was an average gain of 2.0 ± 0.3 ml of red blood cells. On ration A there was a gain of 2.4 ± 0.1 ml in the first 10-day period. For the animals fed diets lacking one of the following: tryptophane, lysine, methio-

nine, histidine, threonine, valine, leucine, isoleucine, and phenylalanine, there were variable gains, or losses. With methionine and threonine absent the gains were 0.9 and 0.8 ml, respectively, not significantly greater than the greatest apparent gain (+ 0.7 ml) in the group of animals on the diet completely lacking protein. Also their paired controls fed the same diets lacking methionine and threonine in the second 10-day period had the largest losses in circulating erythrocyte

TABLE 4

Gains or losses in total circulating red cell volume.

RATION	4 E	CAS	RATION A	RATION A MINUS	RATION A MINUS	RATION A	
PERIOD (days)	0–10	0–10	0–10	10–20	0–10	10–20	
	ml	ml	ml	ml	ml	ml	*The missing amino acid*
	— 0.7	2.4	2.1	— 0.7	0.1	1.5	Tryptophane
	— 0.3	1.7	..	..	0.2	1.1	Lysine
	— 0.3	1.5	2.7	— 1.1	0.9	1.6	Methionine
	— 0.4	2.3	1.9	— 0.2	— 0.2	1.3	Histidine
	0.0	2.4	2.8	— 0.9	0.8	1.9	Threonine
	0.5	2.5	2.0	0.5	0.2	1.8	Valine
	0.7	1.5	1.9	— 0.1	— 0.4	1.3	Leucine
	— 0.2	..	3.0	— 0.6	0.4	1.9	Isoleucine
	..	..	2.8	— 0.6	0.3	1.4	Phenylalanine
	..	..	2.5		1.5[1]	.	Arginine
Ave.	— 0.1	2.0	2.4	— 0.5	0.3	1.5	
S.E.	± 0.2	± 0.3	± 0.1	± 0.2	± 0.1	± 0.1	

[1] The value for arginine-deficient animal was excluded from the average.

volume of all the group. All animals with the exception of the one lacking valine which were fed ration A minus one amino acid in the second experimental period, lost circulating erythrocytes. With valine absent there was a slight apparent gain of + 0.5 ml. The animal lacking arginine again performed quite differently, gaining 1.5 ml of erythrocytes in the first 10-day period. This is somewhat less than the smallest gain of 1.9 ml or than the mean gain of 2.4 ml among the

animals on ration A in the first period. But at 20 and 30 days the gain of T.C.E.V. of the arginine deficient animal equaled that of its paired control (see table 3).

The evidence from the weight gains, food consumption, gains of serum protein and hemoglobin described above, demonstrated that arginine was dispensable for the recovery of the hypoproteinemic rat. Therefore ration C was prepared and fed to 2 animals as described (Frazier et al., '46). The total circulating serum protein and erythrocyte volume changes are tabulated in table 3. The rats fed 9 "essential" amino acids (arginine absent) fabricated serum proteins about as well as the animals fed 16 amino acids over a 10-day interval. Red blood cell volumes in these animals equaled those of the rats on the casein diet but were poorer than those of the animals on ration A. The difference is not statistically significant with so small a number of animals.

DISCUSSION

Several workers have demonstrated that protein hydrolysates can maintain nitrogen balance and induce increases in the concentration of serum proteins in adult humans, children and dogs. Only one group of workers (Madden et al., '43, '44, '45) has systematically attempted to determine the amino acids essential for the production of serum proteins. This group has shown that in the protein-depleted dog the 10 essential amino acids of Rose ('38) will induce and maintain the abundant formation of plasma proteins, and maintain positive nitrogen balance for long periods of time. The deletion of single essential amino acids from their mixtures led in some cases to negative nitrogen balances and weight loss, with continued plasma protein production, in others to sharp decreases in protein production with continued positive nitrogen balances. The omission of arginine (Madden et al., '43) from the diet caused the least disturbance of any of the "essential" amino acids they had tested to that time. There was a slow fall in serum protein production but nitrogen balance was maintained for the 2-week period. This is not dissimilar from the

data presented above except in the behavior of the serum proteins. It is apparent that the rat receiving no arginine in his diet over a period of 30 days gained weight, total circulating protein and erythrocytes at a rate comparable, in fact, almost identical with its paired control. It would appear, therefore, that arginine is not essential for the production of serum proteins or erythrocytes in the adult hypoproteinemic rat. This is borne out by the data on the animals fed 9 "essential" amino acids (arginine absent) as practically the sole amino nitrogen source.

Diet consumption with these amino acid mixtures has been described in paper I of this series. One must, of course, consider the total nitrogen intake in relation to the gains or losses of protein and erythrocytes as well as body weight. As noted in the preceding publication, with a deficiency of one of the essential amino acids diet consumption followed a particular pattern from day to day and was far below that of the complete mixture except in the absence of arginine. Because of the low diet consumptions it is difficult to say exactly what the losses in serum proteins mean. One can calculate the ratio of output of serum protein to intake of protein in those instances where there was a positive output. Thus the mean output of serum proteins in mg gained per gm of protein eaten was 13.2 with a range of 8.1 to 16.1 for the animals on the complete mixture for the first 10-day period. Compared with this the animal lacking tryptophane had a ratio of 4.2, lacking histidine 4.6, threonine 7.3, leucine 12.8, isoleucine 3.3, phenylalanine 12.1. The other animals had negative values for this ratio which, of course, have no real significance. In the case of the individual lack of tryptophane, histidine, threonine and isoleucine this ratio is well below the average for animals fed ration A and less than the performance of the poorest animal in that group. But in the case of the leucine and phenylalanine deficiencies the ratio of production to intake approximates that of the complete diets despite the extremely low intake (3.5 and 2.9 gm of protein ($N_2 \times 6.25$)). In view of the small number of animals on each

deficient diet and the curious behavior of the animals with respect to the diet consumption an evaluation of the biological effects of the deficiency of single essential amino acids must await further study.

SUMMARY AND CONCLUSIONS

Protein-depleted rats can synthesize new plasma protein and erythrocytes on a diet in which the sole source of amino nitrogen is a mixture of crystalline amino acids. The mixture includes tryptophane, lysine, methionine, histidine, threonine, valine, leucine, isoleucine, phenylalanine, and arginine, in relative proportions equivalent to those found by chemical analysis of casein. Small amounts of 6 non-essential amino acids were added to bring the nitrogen level to the equivalent of 9% protein ($N \times 6.25$). Moreover, synthesis occurs to an extent comparable to that produced by a diet containing casein at an equivalent nitrogen level. With synthetic diets identical to the above, but lacking one of the above mentioned amino acids other than arginine there is poor diet consumption and no significant gain in serum protein, just as with the complete absence of protein from the diet. The same is true concerning the production of erythrocytes. On a synthetic amino acid diet entirely lacking arginine a rat can synthesize proteins and erythrocytes to an extent equalling that produced by casein or amino acid mixtures at equivalent nitrogen levels and containing all essential amino acids including arginine. Furthermore, the first mentioned 9 amino acids alone can act as a nitrogen source capable of inducing the formation of serum proteins to an extent comparable to that found with the mixture of 16 amino acids (ration A or "synthetic casein") or the natural protein. Therefore, it can be said that 9 amino acids only are indispensable in the hypoproteinemic rat for the construction of serum protein and erythrocytes.

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RELATIVE ACTIVITY OF THE TOCOPHEROLS IN CURING MUSCULAR DYSTROPHY IN RABBITS [1]

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THREE FIGURES

(Received for publication September 12, 1946)

Nutritional muscular dystrophy in herbivora can be prevented or cured by α-tocopherol, but we can find no reports on the effectiveness of β- or γ-tocopherol relative to the alpha form. This information is essential for 2 reasons: First, the development of a bioassay using dystrophic rabbits or guinea pigs requires such knowledge, and second, the comparative physiology of vitamin E action may be better understood when the relative activity is known of the several tocopherols in curing such apparently unrelated syndromes as fetal resorption of rats and dystrophy of rabbits.

The high urinary excretion of creatine associated with muscular dystrophy rapidly falls to normal when α-tocopherol is given. This observation led Mackenzie and McCollum ('40) and Eppstein and Morgulis ('42) to suggest this as the criterion in a semi-quantitative bioassay procedure for vitamin E. A complicating factor demonstrated by Mackenzie et al. ('41) is the sensitivity of the α-tocopherol requirement of the rabbit to variations in intake of the cod-liver oil used to accelerate the onset of dystrophy. Is this effect due to the highly unsaturated fat acids in the oil? And, if so, would the highly unsaturated fat acids in vegetable oils have a similar action?

[1] Communication No. 101 from the Laboratories of Distillation Products, Inc., Rochester, N. Y.

EXPERIMENTAL

Young male New Zealand White rabbits weighing 600 to 900 gm were transferred gradually over the course of 10 days from a complete diet of ground rabbit chow to a vitamin E-low ration. The composition of the diets is given in table 1. Symptoms of muscular weakness began to appear in 7 to 18 days on either diet 38 or diet 381.

In the work to be reported creatinuria has been adopted as the criterion for the estimation of the severity of muscular dystrophy. Creatine and creatinine were determined daily

TABLE 1

Percentage composition of diets.

	DIET		
	381	38	38-A
Commercial casein	12	15	15
Dry skim milk	15	..	..
Dextrin	36	51	51
Sucrose	10	..	..
Brewer's yeast	8	10	10
Salt mixtures	4	6	6
Cellulose	7	10	10
Lard	6	6	6
Cod-liver oil	2	2	..

by the method of Folin ('14), adapted to the Evelyn colorimeter. Creatinuria is expressed numerically as the ratio of the total creatinine (creatine plus creatinine) to preformed creatinine, rather than as the absolute values for 24-hour excretion. This is essentially the method used by Eppstein and Morgulis ('42) and has the advantage of not requiring a large number of metabolism cages. The procedure assumes that the creatinine excretion is constant and is unaffected by the dystrophic condition. This is not strictly true, since Mackenzie and McCollum ('41) have shown variations in the creatinine excretion curve ranging from a maximum of about 30 mg daily during dystrophy to about 22 mg daily in the normal rabbit. However, with creatine values of 70 to 90 mg

during dystrophy, falling to less than 10 mg daily in the normal rabbit, this slight creatinine variation does not seriously interfere with the ratio method of following the dystrophic condition.

A numerical creatinuria-ratio value of "1" indicates no creatine. A value of less than 1.3 is considered normal. When the ratio value has risen to about 4, a single dose of a curative test substance is given and the creatinuria ratios are determined daily until dystrophy has again set in. A single rabbit is often used for as many as 5 determinations, but deaths are common since it is difficult to determine when a rabbit has gone beyond the response stage. Occasionally it appears that supplement administration precipitates a complete, spastic "paralysis" in which the animal's body is entirely nonflexible and it is unable to raise its head to eat. This condition lasts for several days before death. α-Tocopherol given by mouth or subcutaneous injection of α-tocopheryl phosphate is of no benefit.

The response resulting from a test substance is taken as the number of days between dosage and the time when the creatinuria ratio has reached the same level as at the dosage point. This procedure eliminates some of the effect of creatinine variation, since the animals presumably have the same degree of dystrophy at both time points.

A typical example of the growth and creatinuria response to α-tocopherol is shown in figure 1. A certain degree of correlation can be noted between the growth effects and the creatinuria ratio responses. As pointed out by Mackenzie et al. ('40), creatinuria is the most dependable symptom of vitamin E deficiency in rabbits and precedes both the weight change and the physical symptoms. Although creatinuria may result from causes other than vitamin E deficiency, we feel that this possibility can be ignored here because all rabbits are subjected to at least 1 cure with α-tocopherol.

The tocopherol compounds to be tested were dissolved in U.S.P. olive oil, in such concentrations that between 4 and 8 drops (0.1 to 0.2 gm) delivered from a specially calibrated

dropper would furnish the desired quantity. Calibration was made by weight and by the Emmerie-Engel reaction for fat-soluble reducing substances. The materials tested were natural d,α-, β-, and γ-tocopherol prepared by Dr. Karl Meng in this laboratory, the synthetic dl,α- and γ-tocopherols,[2] and d,α-tocopheryl phosphate of 70% purity used in water solution.

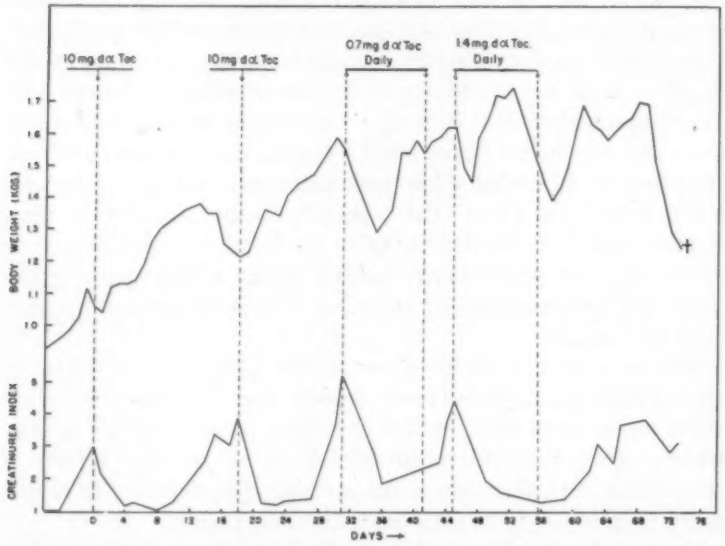

Fig. 1 Typical growth responses and changes in creatinurea index induced by oral administration of α-tocopherol. In some instances vitamin E was fed in a single dose and in others it was fed daily for 2 weeks.

Response to d,α-tocopherol

Several levels of d,α-tocopherol varying from about 2 to 24 mg were fed in single doses to dystrophic rabbits. The creatinuria ratio curves were determined as outlined in the previous section. Dosages are expressed in terms of milligrams per kilogram of body weight at the time of dosage. The responses (days cured) were obtained from the ratio curves.

[2] Merck.

From the results tabulated in table 2 it appears that the responses are a linear function of the logarithm of the dose (mg/kg body weight); on diet 38 the average constant, K, (response/log dose) is 12.5 ± 3.1 (S.D.) while on diet 381 the value is 15.7 ± 1.8 (S.D.). These values correspond with

TABLE 2

Dose-response relations on feeding single doses of d,α-tocopherol to dystrophic rabbits.

BODY WEIGHT	DOSE	LOG DOSE	RESPONSE	K
kg	*mg/kg*		*days*	$\frac{\text{days cured}}{\text{log dose}}$
		A. Diet 38		
0.60	3.54	0.549	5.8	10.6
0.65	3.28	0.516	6.7	13.0
0.64	6.65	0.823	9.0	10.9
0.80	5.30	0.724	13.0	17.9
0.71	6.00	0.778	8.0	10.3
0.55	15.40	1.188	10.5	8.9
0.60	14.20	1.152	13.3	11.5
0.70	12.1	1.083	18.7	17.3
0.95	21.2	1.326	16.6	12.5
0.75	22.6	1.354	21.4	15.8
0.80	21.2	1.326	11.5	8.7
				12.5 ± 3.1 (S.D.)
		B. Diet 381		
1.08	22.20	1.346	24.0	17.8
1.06	9.42	0.974	15.0	15.4
1.21	8.25	0.917	12.2	13.3
1.37	14.60	1.164	17.5	15.0
1.41	4.26	0.629	9.5	15.1
1.07	18.70	1.272	22.8	17.9
				15.8 ± 1.8 (S.D.)

an average daily curative dose of 1.2 and 1.1 mg α-tocopherol, respectively. Diet 38 has been used for the remainder of the work to be reported, but it appears that diet 381 has many points of superiority, chief of which is the smaller coefficient of variation in response and the better growth and general health obtained.

Since the apparent curative dose of α-tocopherol is about 1.1 mg/kg daily, a dystrophic rabbit was given a suboptimal dosage of 0.7 mg of d,α-tocopherol daily for 11 days. As shown in figure 1 the creatinuria declined but was not completely cured. After cessation of dosage the creatinuria rose almost immediately. A larger daily dose (1.4 mg) of α-tocopherol brought about a more effective cure.

TABLE 3

Activity of various tocopherols in terms of d,α-tocopherol equivalence.

BODY WT. OF RABBIT	DOSE	LOG DOSE	DAYS CURED	d,α-TOCOPHEROL EQUIVALENCE	
kg	*mg/kg*			*mg/kg*	%
			dl,α-Tocopherol		
1.05	16.0	1.204	13.0	11.0	68.8
0.95	11.8	1.072	8.6	4.9	41.5
0.80	17.5	1.243	15.4	17.0	97.7
1.20	9.4	0.973	8.3	4.7	48.9
0.92	12.2	1.086	12.2	9.5	77.9
1.30	10.8	1.033	13.0	11.0	101.9
1.04	10.8	1.033	14.6	14.8	136.1
				Average	81.8 ± 12.4 (S.E.)
			d,β-Tocopherol		
0.75	14.7	1.167	7.7	4.2	28.5
0.80	14.0	1.146	7.9	4.3	30.7
				Average	29.6
			d,γ-Tocopherol		
0.60	33.4	1.524	7.7	4.2	12.6
0.65	30.8	1.489	10.5	7.1	23.1
0.60	16.7	1.223	7.2	3.8	22.8
				Average	19.5
			dl,γ-Tocopherol		
0.75	40.0	1.602	3.0	1.7	4.3
0.60	83.2	1.920	8.9	5.2	6.2
1.25	40.0	1.602	7.6	4.1	10.2
				Average	6.9

Response to other tocopherols

Harris, Jensen, Joffe and Mason ('44) noted that dl,α-tocopherol was only 66% as active as d,α-tocopherol in preventing resorptive sterility in rats. A similar difference in the anti-dystrophy potency of these 2 substances has been observed, as shown in table 3; the racemic mixture has, on the average, 82% of the activity of the natural enantiomorph. However, the variations are large. By the statistical "t" test of Fisher the difference is only significant at a level of $P = 0.2$ (80 chances in 100 that the difference is significant). The average K value of 12.5 as obtained is table 1 was used in calculating the results since these rabbits were on diet 38.

The natural d,γ-tocopherol is almost 3 times as active as the synthetic dl,γ-tocopherol in curing dystrophy (table 3). The synthetic γ- has about 8.5% of the activity of synthetic α-, and the natural γ- has about 20% of the activity of the natural α-. A small part of the difference between natural and synthetic γ-tocopherols can be attributed to α-tocopherol remaining in the former.

The natural d,β-tocopherol shows about 30% of the activity of the d,α-tocopherol.

In general, the activity of the various forms of tocopherol shows much the same relationship in the cure of dystrophy in rabbits as in the prevention of resorption in pregnant rats (Harris et al., '44).

Response to d,α-tocopheryl phosphate administered orally and parenterally

Injections of α-tocopherol or α-tocopheryl acetate are of little or no benefit in curing muscular dystrophy in the rabbit (Mackenzie and McCollum, '41; Eppstein and Morgulis, '41). Injections of d,α-tocopheryl phosphate, on the other hand, are somewhat more active than the oral administration of dl,α-tocopheryl acetate (Eppstein and Morgulis, '42).

We have fed and injected d,α-tocopheryl phosphate into dystrophic rabbits with the following results: Orally the

tocopheryl phosphate was 35% more potent than equivalent doses of d,α-tocopherol. When the phosphate was injected intramuscularly cures were obtained, but the creatinuria ratio curves were of such a character as to make quantitative interpretation difficult. Three of these curves are shown in figure 2.

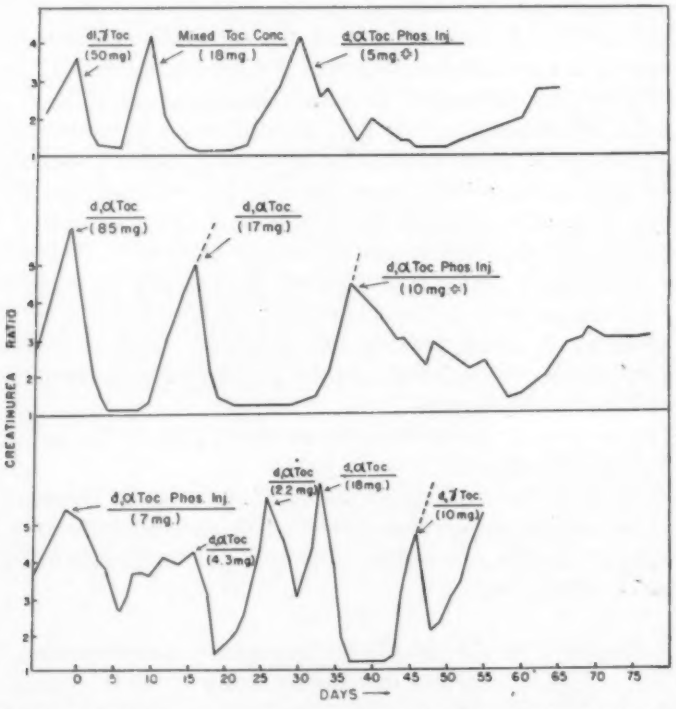
d,γ Toc (50 mg)
Mixed Toc. Conc. (18 mg.)
d,α Toc. Phos. Inj. (5 mg.Φ)
d,α Toc (85 mg.)
d,α Toc (17 mg.)
d,α Toc. Phos. Inj. (10 mg.Φ)
d,α Toc. Phos. Inj. (7 mg.)
d,α Toc (4.3 mg.)
d,α Toc (22 mg.)
d,α Toc (18 mg.)
d,γ Toc. (10 mg.)
CREATINUREA RATIO
DAYS

Fig. 2 Typical response, creatinurea ratios, obtained in rabbits injected with d,α-tocopheryl phosphate.

It will be noted that the urinary creatine falls to a normal level much more slowly than after oral tocopherol administration. Similarly the recurrence of dystrophy is sluggish. In the case of the first 2 animals in figure 2, the gain in body weight during the 30 and 34 days following tocopheryl phos-

phate injection was 300 and 360 gm, respectively. The rabbits then began to lose weight with little or no change in their level of creatine excretion. After becoming extremely emaciated they died by the end of the second month without again developing more than a trace of muscular weakness. There is no way in which their creatinuria ratio curves can be quantitatively interpreted according to the formula used in the previous section. If the recurrence of dystrophy is taken as the point where weight loss sets in, the activity of the injected tocopheryl phosphate may be calculated at 50 and 53 times more active than d,α-tocopherol given orally, and about 6 times more active in the case of the third rabbit of figure 2.

Effect of unsaturated fats on production of dystrophy

The vitamin E requirement of several species is known to be increased by the inclusion of unsaturated fats in the diet. Gottlieb et al. ('43) showed this for rats using tocopherol-free cottonseed oil. Dam ('44) studied the effect of highly unsaturated fractions of hog-liver fat on the vitamin E needs of chickens. Mackenzie and McCollum ('41) have shown that cod-liver oil increases the vitamin E requirement of rabbits and aggravates the dystrophic condition.

Figure 3 illustrates the rate of onset of dystrophy in rabbits as influenced by unsaturated fats, using the creatinuria ratio as the criterion. For this series 38-A was used (diet 38 with the cod-liver oil omitted). Young rabbits were transferred to this diet in the usual way. The various oils tested were fed daily from a hypodermic syringe in quantities of 1.2 ml. All oils were fortified with a concentrate of vitamins A and D to the level of cod-liver oil. The control receiving no oil was fed an equivalent amount of the A and D concentrate by dropper weekly. The oils used were freed of their tocopherols by molecular distillation.[3]

From the results shown in figure 3 it is apparent that the vitamin-free soybean and corn oils and corn oil plus 0.1%

[3] This was kindly done for us by Mr. E. Barnitz of this laboratory.

hydroquinone were as effective as cod-liver oil in inducing dystrophy. The feeding of the oils was stopped on the sixteenth day. It is interesting to note that the creatine excretion dropped toward normal when the oil was stopped, showing more clearly a direct relation of the oils to the condition of dystrophy. After a temporary recession the creatinuria set in again without restitution of the oils.

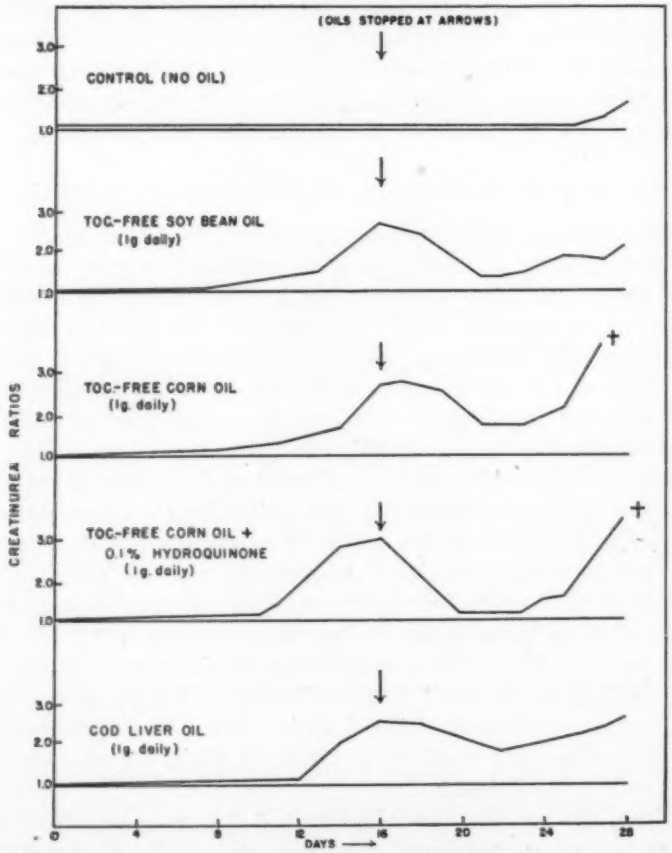

Fig. 3 Changes in creatinurea ratios of rabbits fed 1.2 ml daily of various oils. Unsaturated vegetable fats are as effective as cod liver oil in inducing creatinurea (muscle dystrophy).

DISCUSSION

The natural d,α-, β-, and γ-tocopherols show anti-creatinuria activity in the ratio of 1.0, 0.3, and 0.2, respectively. The synthetic dl,α- and γ-tocopherols are less active than the natural forms. These relative activities are of approximately the same order and degree as has previously been found for the prevention of fetal resorption in rats (Harris et al., '44). The relative activities are also in the same order as their antioxidant potencies in vitro, as determined by Hove and Hove ('44).

The cure of the creatinuria of dystrophic rabbits following injection of d,α-tocopheryl phosphate shows an interesting situation. It appears that the injected tocopheryl phosphate is less readily available to the animal than tocopherol given orally. This is shown by the slow fall of creatine excretion. However, the activity is maintained over a much longer period than would be expected, and recurrence of dystrophy occurs very slowly. The animals may be said to be in a chronic state of dystrophy. Possibly these results indicate that the phosphate is not the physiologically necessary form of the vitamin. An alternate explanation is that the phosphate becomes bound to protein near the site of injection and is liberated slowly over a long period of time. A search for tocopheryl phosphate esterases in animal tissues might throw some light on this situation.

The interrelation of unsaturated fats and tocopherol has been emphasized by showing that the dystrophy-inducing properties of cod-liver oil are shared by tocopherol-free soybean oil and corn oil. The reverse of this interrelation, wherein tocopherols protect or spare essential unsaturated fat acids has recently been shown by Hove and Harris ('46).

In its present form the cure of dystrophy in rabbits does not offer special advantage as a bioassay procedure for vitamin E. Although it is more rapid, it lacks precision and is somewhat more laborious than the fetal resorption method using rats.

SUMMARY

1. The relative activity of natural α-, β-, and γ-tocopherols in the cure of creatinuria associated with muscular dystrophy in rabbits has been found to be 100, 30, and 20, respectively.

2. The minimum curative dose of natural (d) α-tocopherol was 1.1 mg per kilo of body weight per day. For synthetic (dl) α-tocopherol the minimum curative dose was 1.4 mg per kilo per day.

3. The anti-dystrophy potency of synthetic dl,γ-tocopherol was about 30% that of natural d,γ-tocopherol.

4. d,α-Tocopheryl phosphate injected intramuscularly into dystrophic rabbits brings about a slower but longer-lasting cure. The possible significance of this is discussed.

5. Tocopherol-free corn and soybean oils are as effective as cod-liver oil in accelerating the onset of nutritional muscular dystrophy.

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THE ABSORPTION OF RADIOACTIVE IRON BY CHILDREN 7–10 YEARS OF AGE[1]

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TWO FIGURES

(Received for publication September 16, 1946)

Efforts to assess the nutriture of children with respect to iron have usually been limited to studies of hemoglobin concentration and of dietary intakes. Both of these methods have recognized limitations and the interpretation of the data obtained is often controversial. Thus, hemoglobin concentrations below arbitrary limits varying from 10 to 14 gm per 100 ml have been interpreted as signifying anemia (Committee on Diagnosis and Pathology of Nutritional Deficiencies, '43) and dietary intakes less than the Recommended Dietary Allowances have been cited as evidence of iron deficiency states, although these Recommended Allowances were purposely set at a high level and the estimates of allowances for children were based on rather meager data (Food and Nutrition Board, National Research Council, '45).

These considerations have prompted us to organize a pilot study of the absorption of radioactive iron by a group of school children (Darby, Hahn, Steinkamp and Kaser, '46).

[1] This study was supported by grants from the Nutrition Foundation, Inc., the International Health Division of The Rockefeller Foundation, and the Tennessee Department of Public Health.

The data accumulated in this study have not revealed the presence of any considerable degree of iron deficiency among the children studied, but the study has given a new approach to the problem of iron requirements. This study has demonstrated for the first time the feasibility of employing isotopes in large-scale surveys of the nutriture of populations and has led to the institution of a more extensive similar investigation of the iron requirements during pregnancy (Vanderbilt Cooperative Study of Maternal and Infant Nutrition, to be published).

EXPERIMENTAL

Two grade schools for white children in Nashville, Tennessee, were selected to obtain the extremes of economic groupings. School C served a low economic area, School R a high economic district. Some characteristics of the two school districts are compared in the data of table 1 derived from the sixteenth census of the U.S. ('42). The total number of subjects examined were 259 and comprised 88% of the second and third grade pupils in School R and 92% of the pupils in the same grades in School C. All studies were made between January 22 and March 31, 1945.

The following observations were made on each child: a physical examination, laboratory determinations on venous blood of total hemoglobin (Evelyn and Malloy, '38; Rubicon Company, no date), packed cell volume (Wintrobe, '33), serum albumin (micro-Kjeldahl procedure), serum vitamin A (Kaser and Stekol, '43) and ascorbic acid (Bessey, '38), and the calculation of a 7-day food intake record (Steinkamp, Robinson and Kaser, '45). Measurement of the radioactive iron absorption was completed as follows on 188 of the subjects,[2] 176 of whom were aged 7–10 years. A known aliquot of 2–3 mg of ferrous chloride containing Fe^{59}, 47-day half life, reduced

[2] The number of subjects upon whom the iron studies were completed is smaller than the total number of children examined because of unavoidable losses of samples, of inability to complete the studies on some children due to absenteeism, moving from the area, etc. There was no purposeful selection of the subjects for iron absorption studies and no evidence of the occurrence of unconscious selection.

with a slight excess of ascorbic acid, was given orally. This was dispensed in a cup of lemonade and the cup was then rinsed by a second serving of the beverage without added iron. The test dose was administered at least an hour before or after lunch. Two to 4 weeks later another 10-ml sample of venous blood was taken for estimations of the radioactivity

TABLE 1

Population and housing characteristics of school districts R and C, Nashville, Tennessee, according to the U. S. census of 1940.

	CHARACTERISTIC	NUMBER OF INDIVIDUALS		PER CENT OF TOTAL	
		District R	District C	District R	District C
I	Major occupation group				
	Total in occupation group	2,162	4,271	100.0	100.0
	Professional workers	311	57	14.4	1.3
	Semi-professional workers	41	26	1.9	0.6
	Proprietors, managers, officials	534	196	24.7	4.6
	Clerical, sales, and kindred workers	884	742	40.9	17.4
	Craftsmen, foremen, and kindred workers	101	728	4.7	17.0
	Operators and kindred workers	51	1,360	2.4	31.8
	Domestic service workers	152	349	7.0	8.2
	Service workers, except domestic	66	355	3.0	8.3
	Laborers	7	432	0.3	10.1
	Occupation not reported	15	26	0.7	0.6
II	Population by race				
	Total	5,161	13,239	100.0	100.0
	White	4,977	11,456	96.4	86.5
	Colored	184	1,783	3.6	13.5
III	Per cent of dwelling units owner-occupied	....	....	52.7	8.6
IV	Median contract or estimated monthly rent of all dwelling units (dollars)	49.79	13.96	....	....
V	Per cent of dwelling units needing major repairs	....	....	2.5	18.6

of hemoglobin iron and, simultaneously, the packed cell volume and the hemoglobin concentrations were again determined. The estimations of radioactive iron were made as described elsewhere (Hahn, '45; Hahn, Jones, Lowe, Meneely and Peacock, '45).

From the packed cell volume and the body weight data the total red cell mass was estimated by the formula of Balfour, Hahn, Bale, Pommerenke and Whipple ('42):

Body weight in kg × 80 × venous hematocrit reading × 0.75 = Estimated cell mass

The total radioactivity of the circulating hemoglobin iron was then calculated and the percentage absorption of radioactive iron estimated. In this estimation the two assumptions were made that most of the absorbed iron was converted to the iron of the hemoglobin of the circulating red cells and that the iron was not excreted. These assumptions have been demonstrated to be valid for adult iron deficient animals (Hahn and Whipple, '36; McCance and Widdowson, '37; Miller and Hahn, '40; Hahn, Bale, Ross, Hettig and Whipple, '40; Hahn, Bale, Hettig, Kamen and Whipple, '39; Cruz, Hahn and Bale, '42; Moore, Dubach, Minnich and Roberts, '44), although the validity has not been tested for the growing organism.

RESULTS AND DISCUSSION

That there existed a pronounced difference in the economic levels of these two groups of children is apparent from the data of table 1. The children in School R, the economically higher district, had more abundant intakes of calories, protein, vitamins, and minerals than did those of School C. Likewise they showed greater body weights for their ages and higher levels of serum ascorbic acid and carotene (table 2). By all measures the children of School R were abundantly nourished. Despite these differences, the differences in mean hemoglobin concentrations by sex for the ages 7 to 10 are but on the borderline of statistical significance (table 3). The differences by sex at the individual ages are significant only for 10-year old boys and 7-year old girls, although the numbers are small

TABLE 2

Comparison of mean nutritional assessments of children in schools B and C, Nashville, Tennessee, 1945.

ASSESSMENT	SCHOOL B			SCHOOL C		
	No. of children [1]	Sex	Mean ± S.E.	No. of children [1]	Sex	Mean ± S.E.
Per cent of standard weight	60	MF	105.0 ± 1.6	199	MF	97.2 ± 0.7
Calculated daily intake						
Calories	26	M	1931 ± 73	82	M	1842 ± 43
Calories	30	F	1850 ± 58	102	F	1745 ± 38
Protein (gm)	56	MF	68.2 ± 1.7	184	MF	59.5 ± 1.1
Calcium (mg)	56	MF	1193 ± 34	184	MF	889 ± 23
Iron (mg)	56	MF	11.7 ± 0.36	184	MF	10.8 ± 0.19
Total vitamin A (I.U.)	56	MF	6429 ± 436	184	MF	3793 ± 169
Ascorbic acid (mg)	56	MF	67.2 ± 3.7	184	MF	36.6 ± 1.6
Laboratory findings						
Serum ascorbic acid (mg/100 ml)	47	MF	0.814 ± 0.07	187	MF	0.493 ± 0.03
Serum carotene (μg/100 ml)	52	MF	152 ± 7.3	181	MF	96 ± 3.0
Serum vitamin A (I.U./100 ml)	50	MF	93 ± 3.6	172	MF	95.7 ± 2.3
Serum albumin (gm/100 ml)	46	MF	4.96 ± 0.037	190	MF	4.97 ± 0.022

[1] Some of the data on certain children were incomplete; hence, the variations in the numbers.

and there is a general trend toward slightly lower values in School C. We do not feel that the magnitude of these differences is great enough to indicate a physiologically important distinction between the 2 schools. It is apparent from figure 2 that there existed no consistent differences in the radioiron absorption of the children in the 2 schools. Hence, for purposes of further analyses we have combined the data on children from the two schools.

The mean hemoglobin values (table 3) are essentially identical with the means for children in elementary and secondary schools, preparatory boarding schools, and public schools in Great Britain in 1943 (Committee on Haemoglobin Surveys,

TABLE 3

Mean hemoglobin concentrations of children,[1] age 7–10 years, schools C and R, Nashville, Tennessee, 1945.

	SCHOOL R				SCHOOL C			
SEX	Boys		Girls		Boys		Girls	
AGE YEARS	Number	Mean ± S.E.	Number	Mean ± S.E.	Number	Mean ± S.E.	Number	Mean ± S.E.
		gm/100 ml		*gm/100 ml*		*gm/100 ml*		*gm/100 ml*
7	7	13.12 ± 0.156	12	13.33 ± 0.155	14	12.77 ± 0.221	20	12.70 ± 0.150
8	12	13.20 ± 0.223	12	13.18 ± 0.177	26	12.85 ± 0.186	40	12.98 ± 0.120
9	7	13.04 ± 0.163	2	12.95 ± 0.409	28	13.02 ± 0.115	28	12.80 ± 0.145
10	4	13.51 ± 0.402	0	.....	19	12.85 ± 0.135	17	13.16 ± 0.119
7–10	30	13.19 ± 0.093	26	13.23 ± 0.119	87	12.89 ± 0.084	105	12.91 ± 0.066

[1] These data include all of the children studied in this age group. The data of the group upon which iron absorption studies were carried out did not differ significantly from these.

TABLE 4

Absorption of radioactive iron by children, schools C and R, Nashville, Tennessee, 1945.

AGE	7 YEARS		8 YEARS		9 YEARS		10 YEARS	
SEX	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls
Number	17	24	26	34	26	23	17	9
Mean absorption (per cent)	9.26 ± 1.41	7.75 ± 0.78	10.38 ± 1.22	15.84 ± 2.15	16.14 ± 1.01	16.89 ± 2.03	16.68 ± 1.92	14.5 ± 2.4
Median absorption (per cent)	7.75	7.00	8.25	12.00	15.00	13.75	17.75	12.75
Estimated total yearly iron requirement [1] (in mg)	70	67	72	108	152	120	130	163

[1] Data of Heath and Patek ('37).

'45), with the values recorded by Macy ('46) for healthy children, and with the mean levels of similarly aged children in British Columbia (Pett and Hanley, '46), and with those compiled by Wintrobe ('42). It seems unlikely that they reflect any appreciable degree of iron deficiency.

The adult with anemia due to iron deficiency exhibits a greater than usual absorption of iron by the method employed in this investigation (Balfour, Hahn, Bale, Pommerenke and Whipple, '42; Hahn, Jones, Lowe, Meneely and

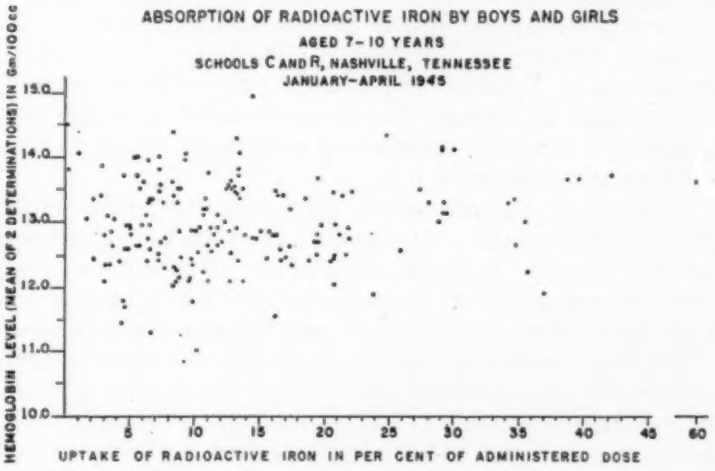

Figure 1

Peacock, '45; Moore, Dubach, Minnich and Roberts, '44; Darby, '46). Thus, hemoglobin concentration and iron absorption may both reflect a body deficit of this element. The lack of correlation between these 2 variables in this study (fig. 1) is interpreted by us as further evidence that the hemoglobin levels as a group were not limited by iron deficiency, and that the means and distributions of hemoglobin concentration found are probably satisfactory for this age group. This conclusion is also supported by the finding that the mean corpuscular hemoglobin concentration for the series was 31.9%

[1] Data of Heath and Patek ('37).

and 32.4% with standard deviations of 1.18 and 0.9, respectively, for Schools C and R. None of these children presented the physical signs of iron deficiency sometimes observed in adults (Darby, '46).

The mean calculated daily iron intake of these children was 11.7 ± 0.36 mg and 10.8 ± 0.19 mg, respectively, for Schools R and C. This level is to be compared with a mean intake of 9.0 mg and 10.7 mg for 7- to 8-year-olds and 10- to 12-year olds, respectively, concluded by Oldham, Roberts and Young ('45) to be adequate. Furthermore, the mean intakes of iron of these children approximated the Recommended Dietary Allowances (Food and Nutrition Board, '45) and the intakes considered by Johnston and Roberts ('42) to be satisfactory for a similar age group. The dietary studies, therefore, also indicated a generally satisfactory nutritional status of these children with respect to iron.

Analysis of the absorption data revealed a remarkable increase in the mean uptake of iron from the 7- to the 9-year groups (fig. 2). Although some of the distributions of absorption are somewhat skewed, the increase is apparent in both the means and medians of each age group and it occurs in the children of both schools. In the 8-year-old group there appeared a significant sex difference in the average per cent of iron absorbed (table 4). The girls absorbed a significantly higher percentage of the administered iron. Upon comparison of these mean absorption values with the estimates of Heath and Patek ('37) for the yearly iron requirements, it is seen that these differences in iron absorption parallel, in general, the estimated yearly increments of body iron. This parallelism is in agreement with the modern theory of iron metabolism (McCance and Widdowson, '37; Hahn, Bale, Lawrence and Whipple, '39). Thus, if the body absorbs iron only as it is needed, the increased requirement during periods of more rapid growth should be reflected in an increased absorption of iron from the gastrointestinal tract at this time. This same proportionality of absorption and need for iron is further illustrated by data on iron absorption during pregnancy (Bal-

four, Hahn, Bale, Pommerenke and Whipple, '42; Vanderbilt Cooperative Study of Maternal and Infant Nutrition, to be published), which indicate that there occurs an increased uptake of iron during the latter part of pregnancy.

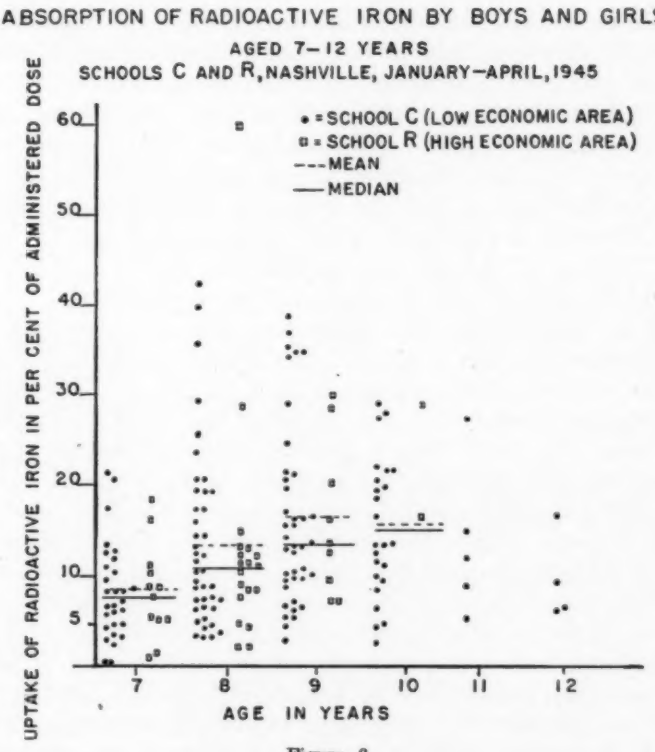

Figure 2

Since the quantity of iron given to these children is approximately that which one might obtain from an ordinary meal, it is possible to estimate from these mean absorption figures the actual mean intake of absorbable iron which is necessary to supply the yearly increment of body iron. This intake divided by 365 gives an estimate of the daily requirement

of absorbable iron. This calculation is illustrated by the formula:

$$\frac{\text{Total yearly gain in circulatory and parenchymal iron}}{365} \times \frac{100}{80} \times \frac{100}{\text{Per cent uptake}} = \text{Mean daily requirement of absorbable iron}$$

The factor $\frac{100}{80}$ is introduced as an allowance for the storage iron, of which the data of Heath and Pátek do not take account, and is an approximation of the storage iron arrived at by Hahn ('37), from analyses of perfused dog tissues. Employing the estimates of Heath and Patek ('37) for the total yearly gain in circulatory and parenchymal iron and applying this calculation, one finds the following estimated mean daily requirements of absorbable iron: for boys 7, 8, 9 and 10 years old 2.6, 2.4, 3.2 and 2.7 mg, respectively; and for girls 7, 8, 9 and 10 years old 3.0, 2.3, 2.4 and 3.8 mg, respectively.

If one assumes that 50% of the dietary iron is absorbable it is obvious that this method of calculation results in a much lower estimate of requirements than the presently accepted allowances. Despite this, it is apparent that these calculations are maximal, for in them it has been assumed that all of the absorbed iron appears in the blood. A correction for iron deposited elsewhere would increase the estimate of absorption and, thereby, reduce the magnitude of the dietary requirement. It is emphasized that these estimates are for the mean dietary requirement of absorbable iron and at present that there exists no reliable means of estimating the quantity of absorbable iron in a dietary; hence, the requirement of total dietary iron cannot now be ascertained by this method. A similar estimate of yearly requirements of body iron based on body weight and the iron content of the tissues of lower animals (Hahn and Whipple, '36) indicates yearly increments approximately twice the magnitude obtained by Heath and Patek. More data on the iron content of human tissues are required before completely reliable estimations of requirements can be made by this method.

It is apparent that iron absorption data such as these may offer a valid means of determining requirements, especially

when quantitative estimates of absorbable iron in foodstuffs are available. The present pilot study demonstrates the possibilities inherent in this approach. The data lead us to suggest that the Recommended Dietary Allowances for iron for children of this age are liberal. It is probable that intakes lower than the present allowances would be adequate if one may assume that at least 50% of the dietary iron is absorbable.

SUMMARY

Determinations of iron absorption on 176 school children, aged 7–10 years, have indicated a mean uptake of 7.75 to 17.75% of a test dose of 2–3 mg of ferrous iron. The median intakes were slightly lower.

The uptake was found to be correlated with the estimated yearly increases of body iron during growth; it was not correlated in these children with other factors tested for, such as economic status of the group, dietary intake as estimated, the hemoglobin level, or general nutritive state.

A new method for the calculation of iron requirements is outlined.

It is estimated from these data that the mean daily requirement of absorbable iron is 2.3–3.8 mg for children of this age group.

It appears that the mean level of approximately 13 gm of hemoglobin in these children was not limited by iron deficiency.

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THE BIOASSAY OF VITAMIN B_6 IN NATURAL MATERIALS[1]

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Recently a more nearly synthetic ration for the assay of vitamin B_6 with the rat has been described and the biological activity of pyridoxine, pyridoxamine and pyridoxal determined under various conditions of administration (Sarma, Snell and Elvehjem, '46). Although these 3 compounds were equally active when fed by dropper as daily supplements to the ration, or when injected intraperitoneally, pyridoxal and pyridoxamine were less active than pyridoxine when the 3 compounds were mixed with the ration. This was also true for the chick. Since these 3 compounds occur naturally, and since vitamin B_6 is ordinarily ingested with the ration, it is clear that microbiological assays with Neurospora sitophila (Stokes, Larsen, Woodward and Foster, '43) or Saccharomyces carlsbergensis (Atkin, Schultz, Williams and Frey, '43) for which pyridoxine, pyridoxal and pyridoxamine are equally active (Snell and Rannefeld, '45), do not necessarily reflect the potency of natural foodstuffs as sources of vitamin B_6 for animal nutrition. In the present paper the vitamin B_6 content of several foodstuffs as determined by rat assay on the new

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ration is given. The influence of certain dietary factors in modifying the activity of pyridoxal and pyridoxine is also considered. Previous values for the vitamin B_6 content of foodstuffs, as determined by animal assay against a pyridoxine standard, have been reported by Conger and Elvehjem ('41), Henderson, Waisman and Elvehjem ('41), Teply, Strong and Elvehjem ('42).

EXPERIMENTAL

The ration employed for the bioassay of vitamin B_6 and the standard curve obtained with graded amounts of pyridoxine HCl have been described previously (Sarma et al., '46). At least 3 animals were used in each group and in many cases a second group of animals was used in order to confirm the results. With each series of experiments, a curve relating weight gain to concentration of pure pyridoxine HCl was obtained and was found to be similar to that described before. The biological materials to be assayed were mixed in the ration and fed at 2 levels estimated to supply between 25 and 75 μg of vitamin B_6 per 100 gm of ration. Weights were recorded weekly and after an assay period of 4 weeks, the average weekly gain of rats in each group was calculated. The vitamin B_6 content of the material under investigation was obtained by reference to the standard curve in each set of experiments.

A variety of biological materials was assayed in this fashion. The results, expressed as pyridoxine hydrochloride, are summarized in table 1. A number of the same samples was assayed by the yeast growth method of Atkin et al. ('43) in an independent investigation dealing with extraction procedures for vitamin B_6 (Rabinowitz and Snell, '46). These values and other comparative values from the literature are also included in the table.

The vitamin B_6 content of the various materials as determined on the improved ration is of the same order of magnitude as that obtained by other workers. For most of the

foodstuffs the assay has been conducted at 2 different levels. The values obtained at 2 levels, although similar, were never identical. Usually, but not always, higher results were found at the lower level of assay. Similar "drifts" have often been encountered in microbiological assays (Neal and Strong, '43).

TABLE 1

Vitamin B_6 content (μg/gm) of biological materials calculated as pyridoxine hydrochloride.

MATERIAL	LOWER LEVEL	HIGHER LEVEL	AVERAGE FIGURE	YEAST ASSAY [1]	VALUES IN LITERATURE
Whole liver powder (Wilson)	...	...	10.0	14.2	
Liver powder 1: 20 (Wilson)	34.3	31.8	33.1	...	45.0 [2], 41.8 [3]
Liver fraction L (Wilson)	15.0	14.0	14.5	21.4	
Brewer's yeast	26.0	23.3	24.7	29.5	
Fleischmann's yeast	40.8	37.8	39.3	44.5	55.0 [2], 39.3 [3]
Duplicate det'n.	43.0	36.0	39.5		
Lamb leg	...	...	4.5	4.6	3.0 [4]
Pork ham	6.3	5.2	5.8	...	5.9 [4]
Beef liver	...	...	8.1	...	7.3 [4], 7.1 [3]
Beef kidney	9.2	11.2	10.2	9.9	4.4 [4]
Wheat germ	11.6	9.0	10.3	11.2	15.9 [4], 9.6 [3], 18.0 [3]
Whole wheat	2.4	2.9	2.7	4.1	4.1 [5], 4.8 [3], 4.6 [6]
Rolled oats	...	...	2.5	1.9	
Rye	...	...	3.7	...	4.3 [6]
Yellow corn	4.5	4.0	4.3	5.7	4.8 [4]
Corn grits	...	...	2.5	...	
Raw milled rice	...	...	1.5		
Soybean flour	7.5	6.7	7.1	8.0	
Split peas	...	...	4.0	1.9	
Vitab	...	...	51.7 [7]	83.8 [7]	
Cerophyl	6.3	6.7	6.5	11.2	8.0 [3]
Skim milk powder	...	...	3.8	...	5.5 [3]

[1] Rabinowitz and Snell ('46).
[2] Conger and Elvehjem ('41).
[3] Atkin, Schultz, Williams and Frey ('43).
[4] Henderson, Waisman and Elvehjem ('41).
[5] Teply, Strong and Elvehjem ('42).
[6] Stokes, Larsen, Woodward and Foster ('43).
[7] The samples assayed by yeast and rat growth methods were not identical in this case.

That the same phenomenon occurs in animal assays has rarely been pointed out, due chiefly to the fact that assays at more than a single level are not always carried out with animals. In the case of a sample of yeast [2] the experiment was repeated for a second time at 2 different levels when similar variation in values was obtained. For many materials the rat assay values were lower than those obtained on the same samples by the yeast method. These lower values obtained by animal assay are to be expected from the observations made before (Sarma et al., '46) that pyridoxal and pyridoxamine when mixed in the ration were less active than pyridoxine, while for yeast each of the 3 compounds possessed equal activity (Rabinowitz and Snell, '46). Snell ('45) found by microbiological assay using different organisms that in yeast, liver extracts and grass juice, most of the active material is in the pyridoxamine fraction. With each of these substances, rat assay gives lower values than does yeast assay. With some other similar materials, however, (e.g., beef kidney, wheat germ), almost identical values are obtained by the two methods. Determinations of the relative distribution of pyridoxal, pyridoxamine and pyridoxine in these materials are not available.

Influence of dietary factors in modifying the activity of pyridoxal and pyridoxine

In view of the reports in the literature that vitamin B_6 is concerned with metabolism of protein (McHenry and Gavin, '41), particularly that of tryptophane (Miller and Baumann, '45) the effect of additions of various individual compounds, mostly amino acids, on the growth promoting powers of suboptimal quantities of pyridoxine and pyridoxal was studied.

Male weanling rats, after a depletion period of 2 weeks on the modified vitamin B_6-deficient diet (Sarma et al., '46) were fed the experimental diets described in table 2. All the diets contained sucrose as the carbohydrate but the protein component differed in the various diets as indicated in the table.

[2] Fleischmann's yeast, R. C. No. 6.

The average weekly gain represents the average increase in weight during the experimental period of 4 weeks.

The addition of dl-methionine at 1% level decreased the growth gain of vitamin B_6-deficient rats from 5 gm per week to only 1 gm per week (groups 1 and 2). A similar growth

TABLE 2

Influence of certain factors in modifying the activity of pyridoxal and pyridoxine in vitamin B_6 deficient rats.

		AVERAGE WEEKLY GAIN AND RANGE
		gm
1.	Vitamin B_6 deficient diet (18% fibrin)	5 (5–6)
2.	Same as 1 + 1% dl-methionine	1 (1–2)
3.	Vitamin B_6 deficient diet (18% casein) + 50 μg/100 gm of pyridoxine HCl	18 (14–23)
4.	Same as 3 + 1% dl-methionine	6 (3–10)
5.	Same as 3 + 0.5% dl-tryptophane	14 (11–16)
6.	Same as 3 + 1% dl-tryptophane	11 (9–12)
7.	Same as 3 + 0.5% dl-tryptophane + 0.5% indole	6 (2–10)
8.	Same as 3 + 1% l-cystine	16 (15–16)
9.	Vitamin B_6 deficient diet (18% casein + 50 μg/100 gm pyridoxal HCl	11 (7–13)
10.	Same as 9 + 1% dl-methionine	3 (2–4)
11.	Same as 9 + 0.5% indole	8 (6–9)
12.	Same as 9 + 0.5% indole + 0.5% tryptophane	3 (2–5)
13.	Same as 9 + 1% l-cystine	12 (9–16)
14.	Same as 9 + 1% dl-alanine	10 (7–12)
15.	Vitamin B_6 deficient diet (18% fibrin) + 50 μg/100 gm pyridoxine HCl	19 (14–24)
16.	Same as 15 + 5% oleic acid	12 (10–15)
17.	Vitamin B_6 deficient diet (18% fibrin) + 50 μg/100 gm pyridoxal HCl	13 (12–15)
18.	Same as 17 + 5% oleic acid	8 (5–11)

depression was observed when dl-methionine or dl-tryptophane or indole were added to rations containing suboptimal amounts of pyridoxine (groups 3 through 7) or pyridoxal (groups 9 through 12). l-Cystine and dl-alanine did not have any significant effect on the activity of pyridoxal or pyridoxine (groups 8, 13 and 14). The addition of oleic acid at a 5% level to rations containing 50 μg or pyridoxine HCl or pyridoxal

HCl per 100 gm of ration resulted in a growth depression (groups 15 through 18). In all cases where growth inhibition was observed, the addition of 250 μg of pyridoxine HCl or pyridoxal HCl per 100 gm of ration counteracted the growth retardation.

DISCUSSION

Miller and Baumann ('43) in studying xanthurenic acid excretion in mice, found that mice on a diet containing 60% casein required 3 times as much pyridoxine as those on 20% casein diet to reduce the excretion of the pigment to the same level. They also found that the addition of l-tryptophane decreased the survival time of mice deficient in pyridoxine but not to the same extent as the addition of casein of equivalent tryptophane content. From these observations it was clear that amino acids other than tryptophane contributed to the ill health of the pyridoxine-deficient mice. However, additions of tyrosine, histidine and phenylalanine to the diet did not affect the growth or survival time of the deficient mice. The experiments reported here on rats confirm the above observations regarding tryptophane in that additions of tryptophane to the diet containing suboptimal amounts of either pyridoxal or pyridoxine resulted in growth retardation. The experiments also suggest that indole acts in the same manner as tryptophane and that dl-methionine is another amino acid whose metabolism may be intimately connected with the function of vitamin B_6.

Several reports have appeared dealing with the interrelationship between vitamin B_6 and unsaturated fatty acids though the exact mechanism is not clearly understood. Birch ('38) showed that vitamin B_6 and unsaturated fatty acids are both involved in acrodynia, while Schneider, Steenbock and Platz ('40) suggested that essential fatty acids, vitamin B_6 and at least 1 other factor were concerned in the cure of the rat dermatitis. Salmon ('41) and Quackenbush, Kummerow, Platz and Steenbock ('42) have reported similar results. The

growth inhibition obtained with oleic acid and the subsequent counteraction with adequate amounts of vitamin B_6 offers additional evidence that vitamin B_6 may be involved in the metabolism of unsaturated fatty acids.

It is quite unlikely that in the estimation of vitamin B_6, tryptophane, methionine or oleic acid will be present in such high concentration as to interfere with the assay for vitamin B_6. However, the possibility cannot be completely ruled out especially with substances of high protein or fat content and low amounts of vitamin B_6. In such cases suitable controls will have to be devised before the estimation of vitamin B_6 can be satisfactorily carried out. It was previously shown (Sarma et al., '46) that in the presence of large amounts of dextrin, rats grew on the ration without added vitamin B_6. This result was attributed to intestinal synthesis of vitamin B_6 under these conditions. It is possible that with substances low in vitamin B_6, but high in starchy materials, influences of this kind might produce erroneously high values.

SUMMARY

Vitamin B_6 in various biological materials was determined with rats, using the modified vitamin B_6-deficient ration (Sarma et al., '46) and the results compared with the values obtained by other methods. The values obtained are in most cases somewhat lower than the values obtained by the yeast growth method, presumably due to the decreased activity of pyridoxamine and pyridoxal when mixed in the ration.

dl-Tryptophane, indole, dl-methionine, and oleic acid have been shown to exert a growth retarding effect when added to rations containing suboptimal amounts of pyridoxal or pyridoxine. This growth inhibition, however, was counteracted by adequate amounts of the vitamin. l-Cystine and dl-alanine were shown to exert no appreciable effect under similar conditions. The possibility of these dietary factors influencing vitamin B_6 estimation is discussed.

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